

**Investigation of the effect of nitrite in the management and treatment of  
myocardial infarction**

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## **Abstract**

Coronary heart disease is the commonest cause of death in the UK, in the main as a consequence of acute myocardial infarction (AMI)) causing 1 in 5 and 1 in 7 deaths in men and women respectively – a total of around 88,000 deaths per year. Presently, timely and effective reperfusion with primary percutaneous coronary intervention (PCI) for AMI remains the most effective treatment strategy for limiting infarct size, preserving left ventricular ejection fraction and improving clinical outcomes. However, the process of reperfusion (known as myocardial ischaemia-reperfusion (I/R) injury) can itself induce cardiomyocyte death, for which there is currently no effective therapy.

A potential solution to the problem of I/R injury may exist in the form of inorganic nitrite ( $\text{NO}_2^-$ ). Over the last decade evidence has accumulated supporting the view that nitrite, which is abundant in blood and tissues, represents a significant stable intravascular endocrine reservoir and tissue storage form of nitric oxide (NO) that exerts a number of beneficial effects. Endogenous NO generation maintains vascular tone, inhibits vascular smooth muscle contraction/growth, platelet aggregation, and leukocyte adhesion to the endothelium. However, during ischaemia the ability of the predominant isoform that underlies this NO synthesis i.e. endothelial NO synthase (eNOS) is severely attenuated as a result of inadequate delivery of oxygen and co-factors. It is clear that identification of strategies that look to restore or replace diminished NO have therapeutic potential.

Extensive preclinical evidence exists to suggest that nitrite (as a source of NO) is an effective therapeutic strategy for preventing myocardial I/R injury. In this project using a randomised clinical trial it was shown whether sodium nitrite reduces I/R injury and subsequent infarct size in patients undergoing primary PCI for AMI, and the mechanisms by which this may have occurred. Additionally the potential long-term benefits of nitrite supplementation post PCI were examined in-vitro models of I/R injury.

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## **Publications**

**Jones DA**, Andiapen M, Van-Eijl TJ, Webb AJ, Antoniou S, Schilling RJ, Ahluwalia A, Mathur A. The safety and efficacy of intracoronary nitrite infusion during acute myocardial infarction (NITRITE-AMI): study protocol of a randomised controlled trial. *BMJ Open*. 2013 Apr 2;3(4). doi:pii: e002813. 10.1136/bmjopen-2013-002813. PMID: 23550096

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## **Abstracts**

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**Jones DA**, Khambata RS, Mathur A, Ahluwalia A. Sodium nitrite promotes the viability and proliferation of endothelial cells but inhibits the growth of smooth muscle cells under hypoxia. *Nitric Oxide* 2013;**31**;S22

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## Abbreviations

AAR	Area at risk
ACEi	Angiotensin converting enzyme inhibitor
ACh	Acetylcholine
AMI	Acute myocardial infarction
ANT	Adenine nucleotide translocase
ATP	Adenosine Triphosphate
AUC	Area under the curve
B/Bo	% bound
BMI	Body mass index
BMS	Bare-metal stent
CABG	Coronary artery bypass grafting
Ca <sup>2+</sup>	Calcium
cGMP	Cyclic guanylate monophosphate
CI	Confidence Interval
CK	Creatine Kinase
CVD	Cardiovascular disease
Cyp-D	Cyclophilin D
DES	Drug-eluting stent
deoxyHb	Deoxyhaemoglobin
deoxyMb	Deoxymyoglobin
ECM	Extra-cellular matrix
FAD	Flavin adenine dinucleotide
FITC	Fluorescein isothiocyanate

Hb	Haemoglobin
HCAEC	Human Coronary artery endothelial cell
HmCASM	Human Coronary artery smooth muscle cell
HUVEC	Human Umbilical vein endothelial cell
IBMX	3-isobutyl-1-methyloxanthine
IHD	Ischaemic heart disease
IS	Infarct size
I/R	Ischaemia-Reperfusion
ISR	In-stent restenosis
LGE	Late Gadolinium enhancement
L-NMMA	L-nitro mono methyl arginine
LVEDV	Left ventricular end-diastolic volume
LVESV	Left ventricular end-systolic volume
MAPK	Mitogen-activated protein kinase
metHb	Methaemoglobin
Mo	Molybdenum cofactor
mPTP	Mitochondrial permeability transition pore
MSI	Myocardial salvage index
MVO	Microvascular obstruction
MI	Myocardial infarction
Mb	Myoglobin
NaF	Sodium fluoride
Na <sub>3</sub> VO <sub>4</sub>	Sodium orthovanadate
NADPH	Nicotinamide-adenine-dinucleotide phosphate
NO	Nitric oxide

$\text{NO}_3^-$	Nitrate
$\text{NO}_2^-$	Nitrite
NOS	Nitric oxide synthase
$\text{ONOO}^-$	Peroxynitrite
$\text{O}_2$	Oxygen
$\text{O}_2^-$	Superoxide
OTW	Over-the-wire
oxyHb	Oxyhaemoglobin
PE	Phycoerythrin
PCI	Percutaneous coronary intervention
PKG	Protein kinase G
RIC	Remote ischaemic conditioning
RISK	Reperfusion injury salvage kinases
ROS	Reactive oxygen species
SAFE	Survivor Activating Factor Enhancement
SD	Standard deviation
SEM	Standard error of the mean
sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
STEMI	ST-segment elevation myocardial infarction
VSMC	Vascular smooth muscle cell
VDAC	Voltage dependent anion channel
WHO	World Health Organisation
XOR	Xanthine Oxidoreductase

## Materials

### Materials

### Manufacturer/City/Country

#### Antibodies

Anti human CD42b	Biolegend, San Diego, USA
Anti-human CD62E/CD62P	Serotec, Oxford, UK
Anti-human CD14-FITC	Becton, Dickinson & company, New Jersey, USA
Anti-human CD16-PE	Becton, Dickinson & company, New Jersey, USA
Anti-human CD16b-PE	Becton, Dickinson & company, New Jersey, USA
Anti-human CD11b-PB	Becton, Dickinson & company, New Jersey, USA
Anti-human CD3-FITC	eBioscience Inc, San Diego, USA
Anti-human CD4-PE-Cy7	eBioscience Inc, San Diego, USA
Anti-human CD8-PerCp-cy5.5	eBioscience Inc, San Diego, USA
Anti-human CD162-APC	eBioscience Inc, San Diego, USA
Anti-human CD62L-APC efluor 780	eBioscience Inc, San Diego, USA

#### Isotype controls

IsoCD11b-PB Mouse IgG1k	Becton, Dickinson & company, New Jersey, USA
IsoCD162-APC Mouse IgG2ak	eBioscience Inc, San Diego, USA
Iso-CD62L-APC efluor 780	eBioscience Inc, San Diego, USA
Iso-CD42b-Mouse IgG1 x	Biolegend, San Diego, USA
Iso-CD62E/CD62P-Mouse IgG1	Serotec, Oxford, UK

## **Solutions/Drugs**

Allopurinol	Sigma-Aldrich Company Ltd, Dorset, UK
CaCl <sub>2</sub> Tablets	Sigma-Aldrich Company Ltd, Dorset, UK
Glacial acetic acid	Sigma-Aldrich Company Ltd., Dorset, UK
HEPES Powder	Sigma-Aldrich Company Ltd, Dorset, UK
Hydrochloric acid	Prolabo VWR, Lutterworth, UK
Immunolyse Solution	Beckman Coulter, California, USA
3-isobutyl-1-methylxanthine	Sigma-Aldrich Company Ltd, Dorset, UK
Isotonic Glucose	Nycomed, Zurich, Switzerland
L-NMMA	Sigma-Aldrich Company Ltd, Dorset, UK
MilliQ NO <sub>x</sub> -free water	Millipore Corporation, Billerica, USA
ODQ	Sigma-Aldrich Company Ltd, Dorset, UK
Paraformaldehyde Powder	Sigma-Aldrich Company Ltd, Dorset, UK
Phosphate Buffered Saline tablets	Sigma-Aldrich Company Ltd, Dorset, UK
Potassium iodide	Sigma-Aldrich Company Ltd., Dorset, UK
Sodium hydroxide	Sigma-Aldrich Company Ltd., Dorset, UK
Sodium nitrate	Sigma-Aldrich Company Ltd, Dorset, UK
Sodium nitrite	Sigma-Aldrich Company Ltd, Dorset, UK
Sodium nitrite (4350M in 10mls) for intra-coronary injection	Ipswich Hospital Manufacturing Unit, UK
Vanadium (III) chloride	Sigma-Aldrich Company Ltd, Dorset, UK

## **Platelet Agonists**

ADP	Chrono-log Corp, Havertown, USA
Collagen	Nycomed Ltd, Zurich, Switzerland

### **Instruments/Equipment**

Haemocytometer	Hawksley, Sussex, UK
Multiplate Aggregometer	Dynabyte Medical, Munich, Germany
Sigma 4KIS Centrifuge	Sigma-Aldrich Company Ltd, Dorset, UK
Multiplate Test Cells	Verum Diagnostica GmbH

### **Cell culture reagents**

Annexin V	Trevigen, Gaithersburg, USA
Annexin binding buffer	Biolegend, San Diego, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich Company Ltd, Dorset, UK
Phosphate buffered solution (PBS)	Sigma-Aldrich Company Ltd, Dorset, UK
Propodium Iodide	Trevigen, Gaithersburg, USA
Trypan blue	Gibco (Paisley, UK)
Trypsin/EDTA	Sigma-Aldrich Company Ltd, Dorset, UK

### **ELISA**

cGMP	GE Healthcare, Little Chalfont, UK
CXCL-1	R&D systems, Minnesota, USA
CXCL-5	R&D systems, Minnesota, USA

### **Flowcytomix beads**

hsCRP	eBioscience Inc, San Diego, USA
MCP-1	eBioscience Inc, San Diego, USA



# **Chapter 1.0**

## **Introduction**

## **1.1 Ischaemic heart disease (IHD)**

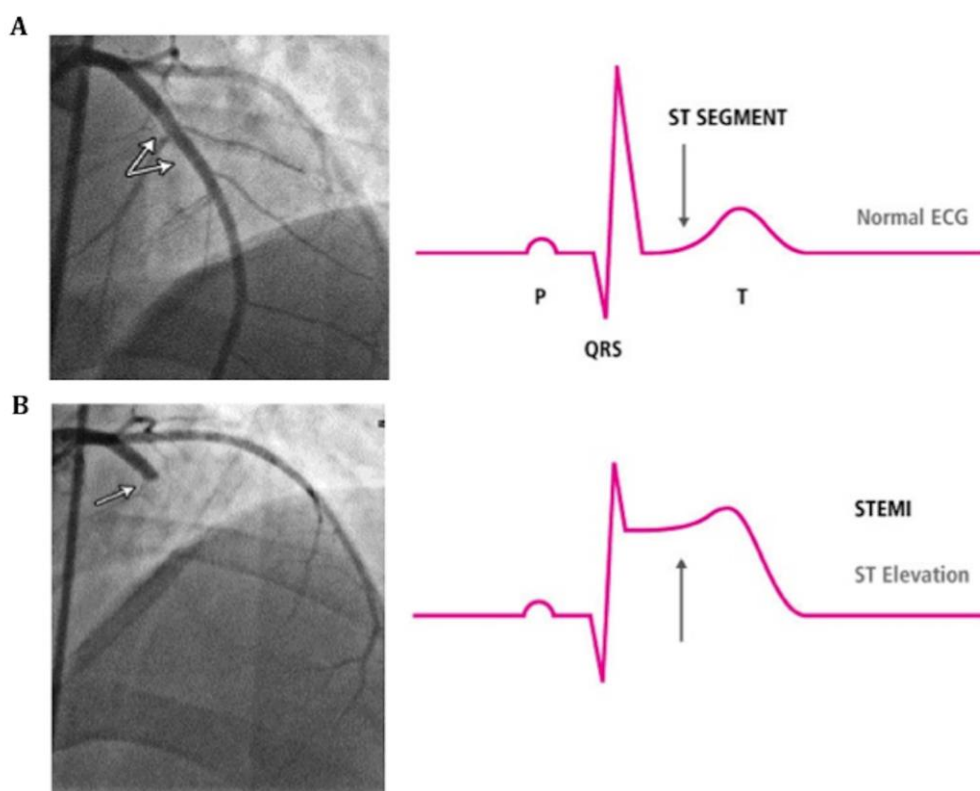
Cardiovascular disease (CVD) is the leading cause of mortality worldwide accounting for 30% of global deaths in 2008, and is likely to remain so until at least 2030 (WHO Cardiovascular disease factsheet 2013). In the United Kingdom (UK) CVD accounts for almost 180,000 deaths each year, with almost half (45%) from IHD and nearly a quarter (27%) from stroke (Townsend et al., 2012). IHD by itself is the most common cause of death in the UK (mainly as a consequence of acute myocardial infarction (AMI)) causing around one in five male and one in eight female deaths respectively– a total of around 80,000 deaths per year. Importantly IHD is the leading cause of premature mortality in the UK causing almost 46,000 premature deaths in 2010. The financial consequences of IHD are considerable, costing the UK economy approximately £6.7 billion per annum in direct health care costs, loss of productivity and informal care, predominantly as a consequence of the morbidity associated with AMI (Townsend et al., 2012).

It is the formation of atheroma, lipid rich deposits, within the epicardial coronary arteries that causes IHD. The reduction in arterial diameter may lead to a slow but progressive increase in symptoms or an atheromatous plaque may rupture suddenly leading to thrombotic occlusion of the coronary artery and the most notable manifestation of IHD, acute myocardial infarction (Thim et al., 2008). ST-segment elevation myocardial infarction (STEMI) is reported as being responsible for 25-47% of all AMI (Mandelzweig et al., 2006; Yeh et al., 2010), accounts for 800 hospital admissions every year per million population in Europe and occurs in approximately 103,000 people in the UK each year (Townsend et

al., 2012). STEMI is distinguished by an abnormal elevation of the ST-segment on the electrocardiogram (ECG) and is the electrical manifestation of an acute occlusion of a coronary artery. ST segment elevation has been recognized as a sign of myocardial ischaemia and damage for nearly 100 years with the 1<sup>st</sup> description back in 1920 by Harold Pardee, with the abnormality described as the *"T wave as being tall and starts from a point well up on the descent of the R wave"*(Pardee, 1920). The ST segment is the portion of the ECG that lies between the end of the S wave (which signifies the end of ventricular contraction) and the beginning of the T wave (signifying repolarisation of the ventricles). It is usually isoelectric (neither positive or negative) with a ST segment considered to be elevated and indicative of myocardial infarction if it is 1mm or more above the baseline (Figure 1.1). As soon as the coronary artery blood supply is interrupted, myocardial damage begins and the longer the blood supply is occluded the greater the amount of heart muscle lost. In animal models of experimental coronary artery occlusion a 'wave-front' of myocardial injury spreads from the endocardium (inner layer of heart muscle) to the epicardium (outermost layer), whereupon the infarction is described as transmural (Reimer et al., 1977). Without urgent treatment this may lead to death from cardiac rupture/arrhythmia/failure or more often will result in substantial morbidity through the development of chronic cardiac failure.

Presently, timely and effective reperfusion with primary percutaneous coronary intervention (PCI) (involving balloon dilatation of the occluded segment with stent insertion) remains the most effective treatment strategy for reducing myocardial infarct (MI) size, preserving left ventricular (LV) ejection fraction, and

preventing the onset of heart failure (Berger et al 1999; Cannon et al., 2000; McNamara et al., 2006; Lambert et al, 2010). However, despite the introduction of primary PCI, and other recent advances such as decreasing symptom to balloon times (total ischaemic time), resulting in a reduction in mortality of ~50% at 30 days (Widimsky et al., 2010), substantial mortality and morbidity rates still persist with respect to longer term outcome (Fox et al., 2010; Menees et al., 2013). In-hospital mortality rates lie at 3–7% depending on the country, whilst 5–6% of patients have a subsequent cardiovascular event by 30 days (Lambert et al., 2010) and 15–20% of patients develop heart failure.



**Figure 1.1 Typical ECG appearance of ST-elevation myocardial infarction**

- a). Normal epicardial coronary arteries and corresponding normal electro-cardio-gram (ECG)
- b). Occluded Left anterior descending coronary artery and consequential ECG displaying ST-segment elevation indicating an ST elevation myocardial infarction.

One of the main determinants of prognosis after AMI is the size of the infarct (Burns et al., 2002; Gibbons et al., 2004; Wu et al., 2008), and importantly increased infarct size is associated with an increased incidence of heart failure and arrhythmias (Majidi et al., 2009; Timmer et al., 2010; Saia et al., 2010). Thus, such statistics together clearly support the need for identification of additional strategies that might decrease infarct size and improve outcome.

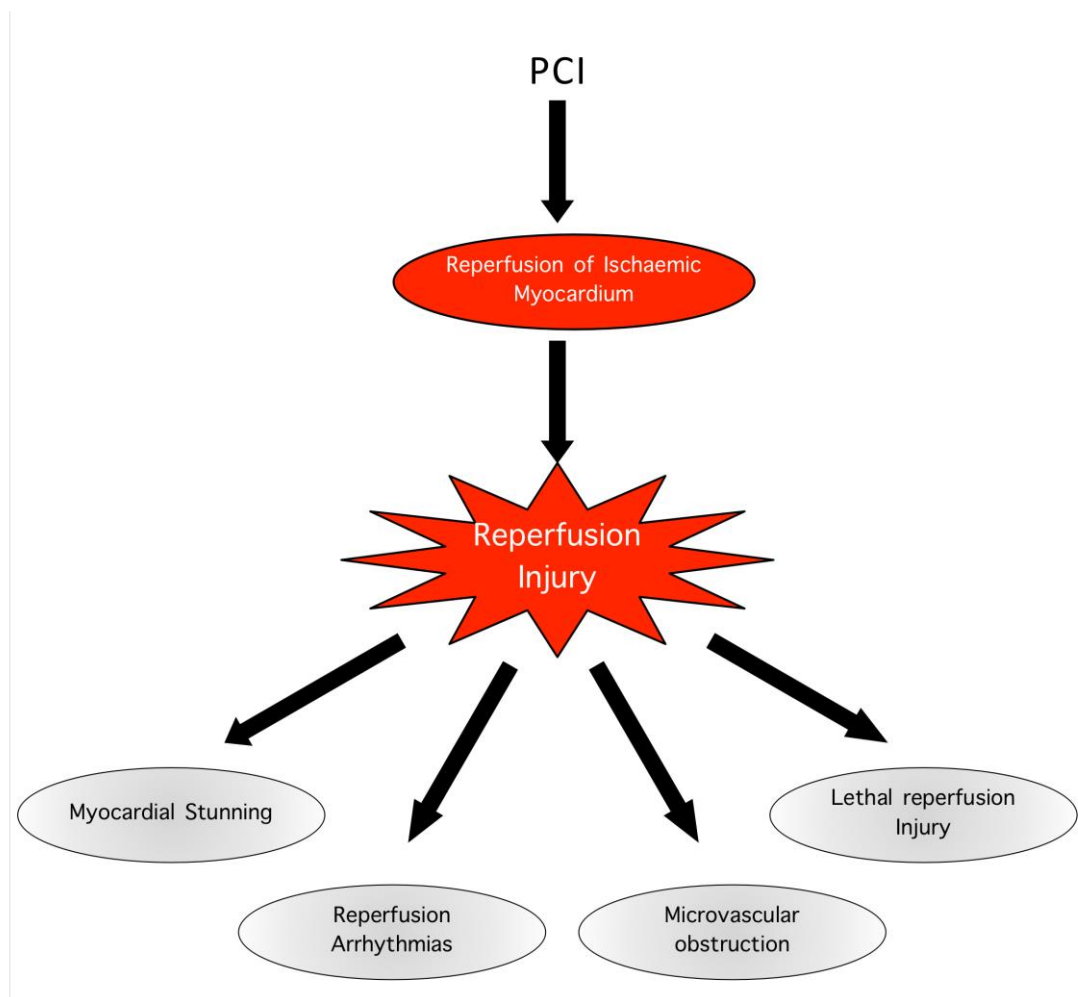
## **1.2 Ischaemia-Reperfusion injury (I/R Injury)**

In the setting of STEMI the immediate reopening of acutely occluded coronary arteries via primary PCI is the treatment of choice to salvage the ischaemic myocardium. However, paradoxically the sudden re-initiation of blood flow, leads to a local acute inflammatory response with further endothelial and myocardial damage. This phenomenon, described as 'reperfusion injury' (Carden and Granger, 2000) may explain why, despite optimum myocardial reperfusion, the 6-month mortality after AMI approaches 12% (Fox et al., 2006) and the incidence of heart failure approaches 15-20% (Lambert et al., 2010; Saia et al., 2010). Animal studies suggest that whilst 50% of the final infarct size is due to the ischaemic insult the remaining 50% is due to reperfusion injury (Yellon and Hausenloy, 2007). Although, the process of myocardial reperfusion continues to be optimized with recent advances in primary PCI technology (thrombus aspiration, novel stents), anti-platelet (Prasugrel, Ticagrelor) and anti-thrombotic therapy (Bivalirudin), there is currently no effective therapy for reducing myocardial I/R injury per se. Therefore, these data underscore the importance of discovering novel therapeutic targets for protecting the heart against acute I/R injury so as to

limit the infarct size, prevent the onset of heart failure, and reduce cardiac mortality.

It is now over 50 years since the first observation that reperfusion following ischaemia was associated with myocardial injury was made by Jennings and colleagues (Jennings et al., 1960). Their report based on experiments in vivo in an open-chest anaesthetised model in dogs subjected to coronary ligation showed that reperfusion appeared to accelerate the development of necrosis, with the histological changes seen following 30-60 minutes of I/R comparable to the degree of necrosis normally seen after 24 hours of permanent coronary occlusion. These histological changes comprised contracture of myofibrils, disruption of the sarcolemma, and the appearance of intra-mitochondrial calcium phosphate particles, features which appeared within minutes of myocardial reperfusion and which differed from those induced by myocardial ischaemia alone, confirming myocardial I/R injury as a distinct pathological entity. Recognition of these findings only became apparent in the mid-1970s, when the significance of I/R injury in reperfused myocardial infarction came into focus in experimental canine models (Mathur et al., 1975). Subsequent work, stimulated initially by both the advent of intracoronary mechanical and thrombolytic reperfusion and the emergence of intravenous thrombolytic therapy, systematically evaluated multiple potential mechanisms of reperfusion injury and explored potential approaches to the prevention or reduction of I/R injury (for reviews see: (Braunwald and Kloner, 1982; Bolli and Marban, 1999; Ito, 2006; Yellon and Hausenloy, 2007).

The injury to the heart caused by myocardial reperfusion causes four types of cardiac dysfunction (Figure 1.2). Some of these are reversible, short lived and easily managed, which include myocardial stunning and reperfusion arrhythmias. However it is the irreversible forms of myocardial reperfusion injury, which include microvascular obstruction and lethal myocardial reperfusion injury, which contribute to the final myocardial infarct size, diminish the benefits of myocardial reperfusion in terms of myocardial salvage and are the main targets for cardioprotection.



**Figure 1.2. Myocardial reperfusion injury results in 4 types of cardiac dysfunction**

**Myocardial stunning.** This is a term which describes the “ mechanical dysfunction that persists after reperfusion despite the absence of irreversible

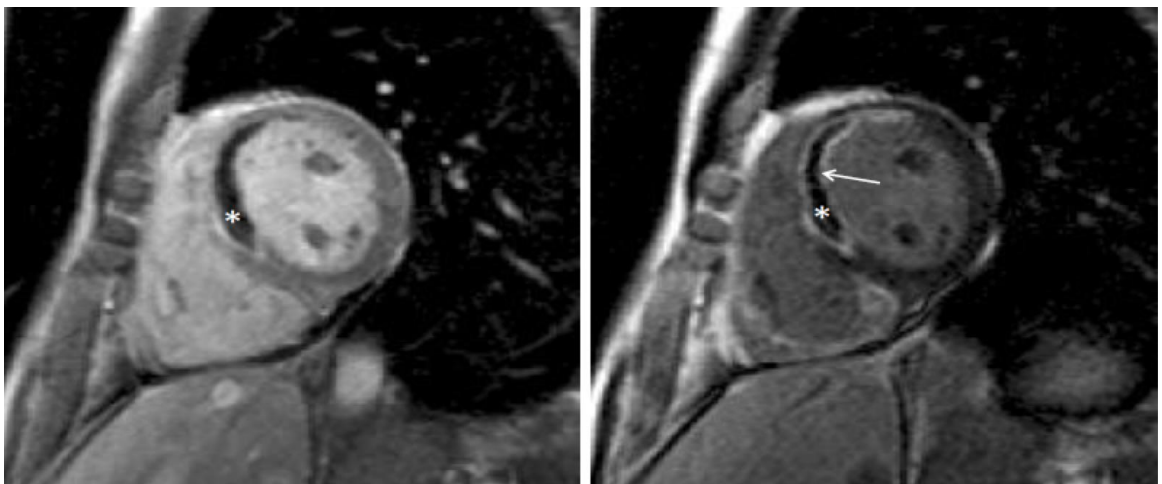
damage and despite restoration of normal or near-normal coronary flow” (Bolli and Marban, 1999) and was initially described by Heyndrickx et al in 1975 (Heyndrickx et al., 1975) In this scenario, reperfusion of either a globally or regionally ischaemic myocardial tissue results in a period of prolonged, yet reversible, contractile dysfunction. The myocardium is essentially “stunned” and requires a prolonged period of time before complete functional recovery.

**Reperfusion arrhythmias.** Experimental animal studies from the 1980s (Manning and Hearse, 1984) were among the first to describe ventricular arrhythmias specifically induced by reperfusion. In reperfused-STEMI patients, the most commonly encountered reperfusion arrhythmias are idioventricular rhythm, ventricular tachycardia, and fibrillation (Kukreja and Janin, 1997). These are usually short-lived and managed easily in the clinical setting (Hausenloy et al., 2013).

**Microvascular obstruction.** Microvascular obstruction was first described in 1966 (Krug et al., 1966) in the feline heart as the “ inability to reperfuse a previously ischaemic region.” Despite intensive research, the underlying cause remains unclear although the major contributory factors are thought to include capillary damage with impaired vasodilatation, external capillary compression by endothelial cell and cardiomyocyte swelling, micro-embolization of friable material released from the atherosclerotic plaque, platelet micro-thrombi, and neutrophil plugging (Ito, 2006). In reperfused STEMI patients it has been reported that the development of the coronary no-reflow phenomenon, the angiographic manifestation of microvascular obstruction, is determined by



several factors which include the extent of myocardial injury, the size of the myocardium at risk of infarction, the patency of the infarct-related artery, (Iwakura et al., 2001) and the presence of thrombus (Yip et al., 2002) in the culprit lesion. Even when post-primary PCI epicardial coronary flow appears normal (TIMI flow 3 (TIMI Study group, 1985)), 30–40% of these patients actually have evidence of microvascular obstruction when undergoing cardiac imaging such as cardiac magnetic resonance (CMR) imaging (Figure 1.3) (Bogaert et al., 2007). The presence of microvascular obstruction in reperfused-STEMI patients is associated with a larger MI size, worse LV ejection fraction, adverse LV remodelling, and worse short-term and long-term clinical outcomes (Ito, 2006; Bogaert et al., 2007).



**Figure 1.3. Microvascular obstruction.**

The left panel displays early gadolinium enhancement revealing evidence of microvascular obstruction (\*) and the right panel, late gadolinium enhancement depicting a transmural myocardial infarct (arrow) with a core of microvascular obstruction (\*).

**Lethal myocardial reperfusion injury.** Lethal myocardial reperfusion injury refers to the reperfusion-induced death of cardiomyocytes, which were viable or reversibly injured at the end of ischaemia (Piper et al., 1998). The existence of lethal myocardial reperfusion injury as a distinct entity, which is capable of independently inducing cardiomyocyte death following a sustained episode of myocardial ischaemia, has been hotly debated over the years (Braunwald and Kloner, 1985; Kloner 1993; Piper et al., 1998). Part of the problem has been the inability to directly demonstrate that the actual process of reperfusion induces the death of cardiomyocytes, which were viable at the end of the ischaemic episode. However, the most convincing means of showing the existence of lethal reperfusion injury as a distinct mediator of cardiomyocyte death is to show that the myocardial infarct size can be reduced by an intervention used at the beginning of myocardial reperfusion. Now extensive indirect evidence has been provided by a large number of pre-clinical animal studies demonstrating 40–50% reductions in MI size with therapeutic interventions applied at the onset of myocardial reperfusion (For review, see Yellon and Hausenloy, 2007).

### **1.3 Mechanisms of Lethal Reperfusion Injury**

In vitro and in vivo experimental data strongly suggest that reperfusion injury is initiated during the first few moments of reperfusion and proceeds over minutes to hours in a cascade involving numerous interactive mechanisms leading to damage or death of myocardium and coronary vascular endothelium (Piper et al., 1998). This reperfusion injury contributes to contractile dysfunction, metabolic derangements, and ultimately cell death by necrosis, apoptosis, and autophagy.

The mechanisms underlying lethal reperfusion injury include: (1) generation of reactive oxygen species (oxygen paradox); (2) intracellular calcium accumulation (calcium paradox); (3) rapid normalization of intracellular pH (pH paradox); (4) an inflammatory response and (5) opening of the mitochondrial permeability transition pore (mPTP).

### **1.3.1 Generation of Reactive oxygen species (ROS) (Oxygen paradox)**

ROS are short-lived oxygen-derived free radicals, which act as signaling molecules and play important roles in biological processes at low concentrations. However, excessive amounts of ROS can damage or destroy organelles, cells and tissues leading to an array of pathologies including DNA mutations possibly underlying cancer, parkinsons disease, ageing and atherosclerosis. Importantly, there is an abundance of evidence that the overproduction of ROS is involved in the pathophysiology of myocardial I/R injury. Molecular oxygen, when reintroduced into a previously ischaemic myocardium, undergoes a sequential reduction leading to ROS formation such as superoxide anion ( $O_2^-$ ), hydroxyl radical (OH), and peroxynitrite ( $ONOO^-$ ) within the first few minutes of reflow (Bolli et al., 1989). In 1973, Hearse and colleagues reported that the reoxygenation of globally arrested (hyperkalemic) anoxic hearts for 100 minutes was accompanied by a release of creatine phosphokinase indicative of cell death, a phenomenon later called the 'oxygen paradox' (Hearse et al., 1973). The exogenous administration of oxygen radical generators has since replicated the myocardial morphological damage and contractile dysfunction observed after I/R injury (Ytrehus et al., 1986; Bolli et al., 1989), with subsequent studies using

oxygen free radical scavengers confirming this explosive cell necrosis is caused by ROS (Lucas et al., 1980).

The major sources of ROS in the ischaemic heart include the enzyme xanthine oxidase, the mitochondrial electron transport chain, and the NADPH oxidase system (for review, see Zweier and Talukder, 2006). Xanthine oxidase has been particularly implicated as a generator of free radicals in the reperfused heart, as its substrates (NADH) accumulate during ischaemia (Thompson-Gorman and Zweier, 1990). Oxidative stress may increase by as much as 100-fold during ischaemia and reperfusion, due to significant acceleration in ROS production (Zweier et al., 1987; Zweier and Talukder, 2006).

The formation of ROS causes damage to cardiomyocytes and vascular tissue, especially the coronary vascular endothelium, which is the 'front line' defense against destructive processes derived from the intravascular space. These ROS are highly reactive and quickly overwhelm the cell's endogenous free radical scavenging system including superoxide dismutase, catalase, glutathione peroxidase and uric acid (Vaage et al., 1997). This, in turn, triggers cellular injury by reactions with lipids, proteins, and nucleic acids. The deleterious actions of ROS on cells and tissues includes damage to DNA (Toyokuni, 1999); lipid peroxidation leading to membrane damage and calcium ( $\text{Ca}^{2+}$ ) overload (Steinberg, 2013); damage to the sarcoplasmic reticulum leading to  $\text{Ca}^{2+}$  dyshomeostasis and contractile dysfunction (Rowe et al., 1983); denaturation of proteins, enzymes and ion channels (Zweier and Talukder, 2006); impairment of metabolism i.e. glycolysis; activation of adhesion molecules on vascular

endothelium thereby stimulating an increase in PMN adhesion (Serrano et al., 1996) and acting as chemoattractants to neutrophils and other inflammatory cells resulting in white cell plugging of capillaries and microvascular obstruction (Zimmet and Hare, 2006). In addition to damaging nuclear and cytosolic elements, ROS can trigger the opening of the mPTP (Zorov et al., 2009), which results in cell death by necrosis, further details of which are described in section 1.3.5.

Importantly ROS reduce the bioavailability of NO, which has far-reaching effects on the myocardium. Under normal conditions NO generation elicits vasodilation (Moncada and Higgs, 1993; Quyyumi et al., 1995), but also exerts a number of other beneficial, protective effects during I/R, likely by influencing oxygen consumption (Clementi et al., 1999), inhibiting platelet aggregation (Radomski et al., 1987), inhibiting leukocyte adhesion (Kubes et al., 1991; Ahluwalia et al., 2004) and is also important for free radical scavenging (Beckman et al., 1990).

NO levels are repressed directly by the reaction of ROS with NO in particular to form the potent oxidant ONOO<sup>-</sup> which itself may cause further damage (Wang and Zweier, 1996). ONOO<sup>-</sup> is believed to react with and damage many important biological molecules including thiols, lipids, proteins, and nucleic acids by a number of mechanisms (Pryor and Squadrito, 1995; Wang and Zweier, 1996). However there has been some controversy with regards to whether ONOO<sup>-</sup> is harmful or protective in the setting of I/R injury (Grisham et al., 1998; Ronson et al., 1999; Ferdinandy and Schulz, 2001). It is now generally accepted that it is likely that ONOO<sup>-</sup> produced within the myocardium is cytotoxic and contributes

to I/R injury but exogenously produced or infused ONOO<sup>-</sup> appears to be protective mainly via its reaction with thiols to generate nitrosothiols and related products which exert anti-neutrophil actions (Ferdinandy and Schulz, 2001).

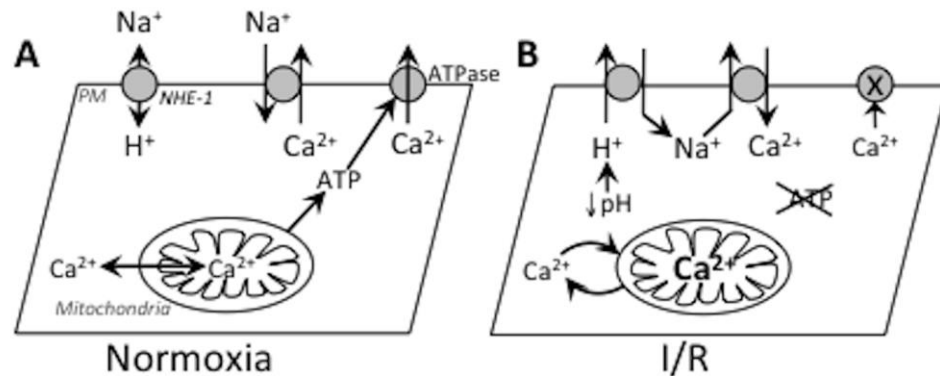
Additionally ROS can alter nitric oxide synthase (NOS) function (the primary family of enzymes implicated in endogenous NO generation) inducing a switch from being an NO to O<sub>2</sub><sup>-</sup> generator (See later section 1.5.2). All 3 NOS isoforms can become potent sources of O<sub>2</sub><sup>-</sup> with depletion of either the substrate L-arginine or the co-factor tetrahydrobiopterin (BH<sub>4</sub>) triggering this fundamental alteration in NOS function (Xia et al., 1996; Xia et al., 1998; Chen et al., 2010) (see later section 1.5.2). Since BH<sub>4</sub> can be readily depleted by oxidants this results in 'uncoupling' of NOS switching it from NO to O<sub>2</sub><sup>-</sup> generation. Other mechanisms that could trigger uncontrolled O<sub>2</sub><sup>-</sup> generation include the release of FAD from the NOS enzyme and disruption of dimerization, which is critical for NO generating capacity (Xia and Zweier, 1997). This increased formation of O<sub>2</sub><sup>-</sup> rapidly removes NO to form ONOO<sup>-</sup>, and this can then itself or via ONOO<sup>-</sup> oxidize BH<sub>4</sub> that has not been removed during ischaemia. The effects are two-fold, firstly this results in a self-sustaining of the production of O<sub>2</sub><sup>-</sup> resulting in a vicious cycle (Ma et al., 1993) but also depression of NO generation. This reduction in availability of endogenous NO may contribute to the pathogenesis of MI since inhibiting NO or its production is associated with larger infarct sizes (Johnson G III et al., 1991; Jones et al., 1999).

### **1.3.2 Intracellular $\text{Ca}^{2+}$ accumulation ( $\text{Ca}^{2+}$ paradox)**

It was first reported in the 1970s that reperfusion following brief coronary occlusion was associated with accelerated accumulation of intracellular  $\text{Ca}^{2+}$  (Shen and Jennings, 1972). It is now well established that at the time of myocardial reperfusion, there is an abrupt increase in both intracellular and mitochondrial  $\text{Ca}^{2+}$  which overwhelms the normal mechanisms that regulate  $\text{Ca}^{2+}$  in the cardiomyocyte; this phenomenon is termed the  $\text{Ca}^{2+}$  paradox (Zimmerman and Hulsman, 1966). The accumulation of intracellular  $\text{Ca}^{2+}$  during ischaemia occurs in response to the loss of energy homeostasis and as a result of changes in the acute regulation of sarcolemmal and sarcoplasmic reticulum cation transport mechanisms (Figure 1.4). Specifically during ischaemia the depletion of adenosine triphosphate (ATP) results in subsequent inhibition of the energy-dependent sarcolemmal  $\text{Na}^+/\text{K}^+$  ATPase, increased activity of  $\text{Na}^+-\text{H}^+$  exchanger and reverses the activity of the  $\text{Na}^+-\text{Ca}^{2+}$  exchanger (Inserre et al., 2005). ROS adversely influences  $\text{Ca}^{2+}$ -handling proteins in the sarcoplasmic reticulum as described in section 1.3.1 which leads to higher levels of cytosolic  $\text{Ca}^{2+}$  and facilitates  $\text{Ca}^{2+}$  entry through L-type channels and the entry of  $\text{Ca}^{2+}$  into the cell due to a loss of membrane integrity (Bersohn et al., 1997).

The physiological consequences of this  $\text{Ca}^{2+}$  accumulation are arrhythmias, hypercontracture of the cardiomyocytes causing cell death and/or opening of the mPTP (Minezaki et al., 1994) which leads to cell death by necrotic or apoptotic pathways. In addition, the contractile apparatus becomes desensitized to  $\text{Ca}^{2+}$ ,

thereby impairing the contractile function of the myofibrils (Piper et al., 1998; Nakanishi et al., 1991) and changes in left ventricular ejection fraction after AMI (Urquhart et al., 1985; Urquhart et al., 1984).



**Figure 1.4 Schematic of  $\text{Ca}^{2+}$  paradox in myocardial I/R injury**

Panel A displays the normal pathways controlling cellular  $[\text{Ca}^{2+}]$ . Panel B displays the events leading up to  $[\text{Ca}^{2+}]$  overload during I/R. A drop in pH triggers  $\text{Na}^+/\text{H}^+$  exchanger mediated  $\text{Na}^+$  influx, resulting in  $\text{Na}^+$  overload. Reverse-mode  $\text{Na}^+/\text{Ca}^{2+}$  exchange across the plasma membrane then results in  $\text{Ca}^{2+}$  overload and subsequent mitochondrial  $[\text{Ca}^{2+}]$  overload.  $\text{Ca}^{2+}$  cycling across the mitochondrial membrane can lead to propagation  $\text{Ca}^{2+}$  export by  $\text{Ca}^{2+}$ -ATPases. Adapted from (Brookes et al., 2004)

### 1.3.3. Rapid normalization of intracellular pH (pH Paradox)

The rapid restoration of physiologic pH during myocardial reperfusion, which follows the washout of lactic acid and the activation of the  $\text{Na}^+/\text{H}^+$  exchanger, contributes to lethal I/R injury. This phenomenon is termed the pH paradox (Lemasters et al., 1996). Both interstitial and tissue pH rapidly decrease with onset of ischaemia. This tissue acidosis inhibits the potentially deleterious effects of hypercontracture (Ladilov et al., 1995), cell-cell communication by gap junctions, calpain activation,  $\text{Na}^+/\text{H}^+$  exchanger and opening of the mPTP (Piper et al., 2004; Kim et al., 2006). However, reperfusion rapidly restores tissue pH by



washout of  $H^+$  from the interstitial space, which then unleashes the aforementioned mechanisms to cause cell injury. In neonatal rat cardiomyocytes, experimental studies have shown that reoxygenation with acidic buffer is cardioprotective, an effect that may be mediated by the inhibition of mPTP opening (Bond et al., 1991).

Alternative strategies such as administration of  $Na^+$ - $H^+$  exchanger inhibitors have been shown to be cardioprotective before ischaemia whereas administration specifically at reperfusion has not shown a consistent cardioprotective effect with some studies showing benefit (Sato and Kitada, 2004; Gumina et al., 1998) and others not (Klein et al., 1995). The failure of  $Na^+$ - $H^+$  exchanger inhibitors to reduce infarct size when administered at reperfusion may be related to the contribution of other mechanisms to reperfusion injury such as  $Ca^{2+}$  overload.

#### **1.3.4. Mitochondrial permeability transition pore (mPTP)**

Mitochondria are known for being the powerhouse of cells, generating ATP through oxidative phosphorylation. However in mammalian mitochondria it has been increasingly acknowledged that a latent non-specific pore within the inner mitochondrial membrane, known as the mPTP, plays a pivotal role in cell death during reperfusion. It was originally discovered back in the 1970s (Haworth and Hunter, 1979) however its critical involvement in myocardial I/R injury was revealed a decade later (Crompton et al., 1987). In studies using isolated cardiac myocytes (Nazareth et al., 1991b; Leyssens et al., 1996) and Langendorff perfused hearts (Griffiths and Halestrap, 1993; Griffiths and Halestrap, 1993) it was

demonstrated that mPTP opening occurs during reperfusion, and that preventing this opening provides protection against I/R injury. These pioneering studies were slow to be appreciated however and it is only in recent years that data has accumulated from many different laboratories confirming the central role of mPTP opening in reperfusion injury and its importance as a pharmacological target for cardioprotection (Weiss et al., 2003; Halestrap, 2009; Perrelli et al., 2011).

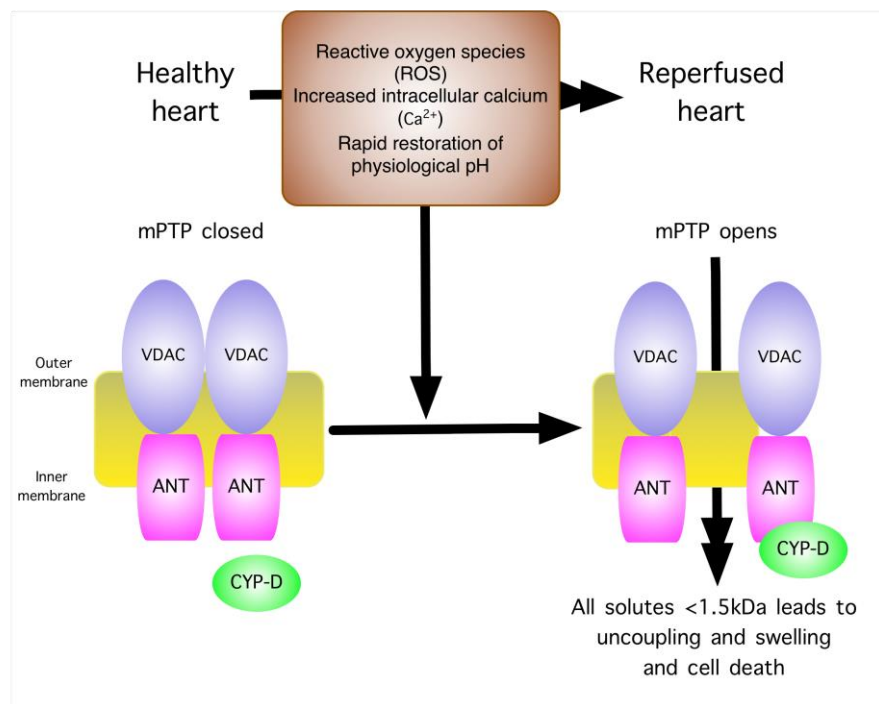
Despite extensive studies, the identity of the pore-forming proteins of the mPTP are still debated. Until recently, three molecules were accepted as key structural components of the mPTP, including adenine nucleotide translocase (ANT) in the inner membrane, cyclophilin D (Cyp-D) in the matrix, and the voltage dependent anion channel (VDAC), also known as porin in the outer membrane. However it appears that some of these components (VDAC and ANT) are in part dispensable (Baines, 2010) with CypD being the only essential regulator of pore opening, and the only genetically proven indispensable mPTP component with CypD knockout mice shown to be protected from I/R injury (Baines et al., 2005; Baines et al., 2007).

Under physiological conditions the mPTP remains closed and due to inhibition by cellular acidosis the pore remains quiescent during ischaemia (Griffiths and Halestrap, 1995). The pore is sensitive to a number of stimuli; alterations in  $\text{Ca}^{2+}$  concentration, generation of ROS, restoration of physiological pH and the presence of inorganic phosphate (Lapidus and Sokolove, 1994), conditions, all of which are created within a few moments of reperfusion. Opening of the pore

results in it becoming freely permeable to solutes of up to 1.5 kDa including protons (Massari and Azzone, 1972; Haworth and Hunter, 1979) (Figure 1.5). The crossing of protons results in a rapid dissipation of the electrical gradient across the membrane, uncoupling of oxidative phosphorylation (failure of ATP generation) and reversal of the ATP synthesis machinery catalyzing the hydrolysis of ATP produced by glycolysis, resulting in a rapid decline in cellular ATP concentration (Halestrap et al., 2004). If the pore remains open, this leads to cell death by necrosis. At the same time, as proteins are unable to cross the membrane, an osmotic gradient is generated causing the mitochondria to swell, rupturing the outer mitochondrial membrane and releasing cytochrome C (Zoratti and Szabo, 1995). Cytochrome C then associates with apoptosis inducing factor and caspase-9, which induces caspase-3 release and apoptosis. The pro-apoptotic members of the Bcl-2 (B-cell lymphoma-2) family are also up-regulated and contribute to apoptotic cell death in this setting (Gustafsson and Gottlieb, 2003).

Importantly, the mPTP remains closed during myocardial ischaemia and opens only during the first few minutes of reperfusion (Griffiths and Halestrap, 1995) making it an ideal target for therapeutic manipulation in the setting of AMI. In accordance with mPTP opening in the early minutes of reperfusion cardioprotection from I/R injury using mPTP inhibitors cyclosporine A, NIM811 or sanglifehrin A given at reperfusion have been reported both in vitro and in vivo (Griffiths and Halestrap, 1993; Griffiths and Halestrap, 1995; Hausenloy et al 2003) and by inhalational anaesthetics that inhibit pore opening (Ge et al., 2010). Furthermore, mice lacking cyp-D have been reported to sustain smaller

myocardial infarcts than control animals (Baines et al., 2005). Additionally the pharmacological inhibition of the mPTP in human atrial trabeculae subjected to simulated I/R injury also exhibit cardioprotection (Shanmuganathan et al., 2005). This inhibition of mPTP opening is believed to partly underlie the mechanism of several proposed cardioprotective mechanical “conditioning” interventions including ischaemic pre-conditioning and postconditioning (Hausenloy et al., 2004) (Argaud et al., 2005).



**Figure 1.5. Proposed Molecular Mechanism of the mitochondrial permeability transition pore (mPTP).**

VDAC= voltage dependent anion channel, ANT= adenine nucleotide translocase, Cyp-D= cyclophilin D.

#### 1.3.4. Inflammation

Inflammation is an important contributor to the pathophysiology of myocardial I/R injury and hypotheses pertaining to pathological mechanisms are an evolving

area of intense research and debate. The classic inflammatory response to acute ischaemia is predominantly an innate immune response (Entman and Smith, 1994). Polymorphonuclear cells have been shown to be the major leukocytes found in necrotic tissue following ischaemic injury with neutrophils the early cellular mediators of local microvascular changes and parenchymal damage (Engler et al., 1986a; Engler et al., 1986b; Vinten-Johansen, 2004). Monocytes and macrophages infiltrate later and extend the early injury phase (Ysebaert et al., 2000). The complement system is also activated and contributes to tissue destruction (Hill and Ward, 1971; Vakeva et al., 1994).

More recently a role for an adaptive immune response has also been implicated in the pathogenesis of I/R injury with recent data suggesting that lymphocytes have a significant role in the response to I/R injury in a variety of organ systems (Linfert et al., 2009). Lymphocyte-related cytokines are upregulated in the post-ischaemic heart, (Herskowitz et al., 1995; Squadrito et al., 1999) and leukocyte adhesion molecules and chemokines, such as P-selectin glycoprotein ligand-1, CD11/CD18, intercellular adhesion molecule-1 (ICAM-1), and monocyte chemoattractant protein-1 (MCP-1), mediate experimental myocardial I/R injury (Entman et al 1992; Herskowitz et al., 1995; Birdsall et al., 1997) and lymphocyte adhesion, migration, and signaling (Loetscher et al 1996; Siveke and Hamann, 1998).

The initiating stimulus activating the immune system is the myocardial damage (Taqueti et al., 2006). Shortly after ischaemia as a consequence of cell necrosis and apoptosis, the damaged tissue exposes ligands (e.g. non-myosin heavy chain

type IIA and C) that are recognised by components of the innate immune system and leads to its activation (Zhang et al., 2004). After the restoration of coronary blood flow, the activation of complement, release of cytokines and secretion of ROS from endothelial cells promote the infiltration of neutrophils into the myocardium. Complement fragments (e.g. C5a) (Ivey et al., 1995) and cytokines (Tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Richter et al., 1990), Interleukin (IL)-1, 6 and 8 (Kukielka et al., 1995; Smith et al., 2006), platelet activating factor (PAF) (Montrucchio et al., 1993), CXCL-1 (also known as KC) (Miura et al., 2001; Tarzami et al., 2003; Mersmann et al., 2010), CXCL-2 (also known as macrophage inflammatory protein-2 (MIP-2) (Chandrasekar et al., 2001; Tarzami et al., 2003) and CXCL-5 (Chandrasekar et al., 2001)) act as activating or chemoattractant factors that stimulate neutrophils to accumulate within the ischaemic-reperfused myocardium (Vinten-Johansen, 2004). Cytokine generation in the myocardium is regulated at the transcriptional level by NF- $\kappa$ B, which is activated by cytokines themselves or by ROS (Chandrasekar and Freeman, 1997; Fan et al., 2002) (Figure 1.6).

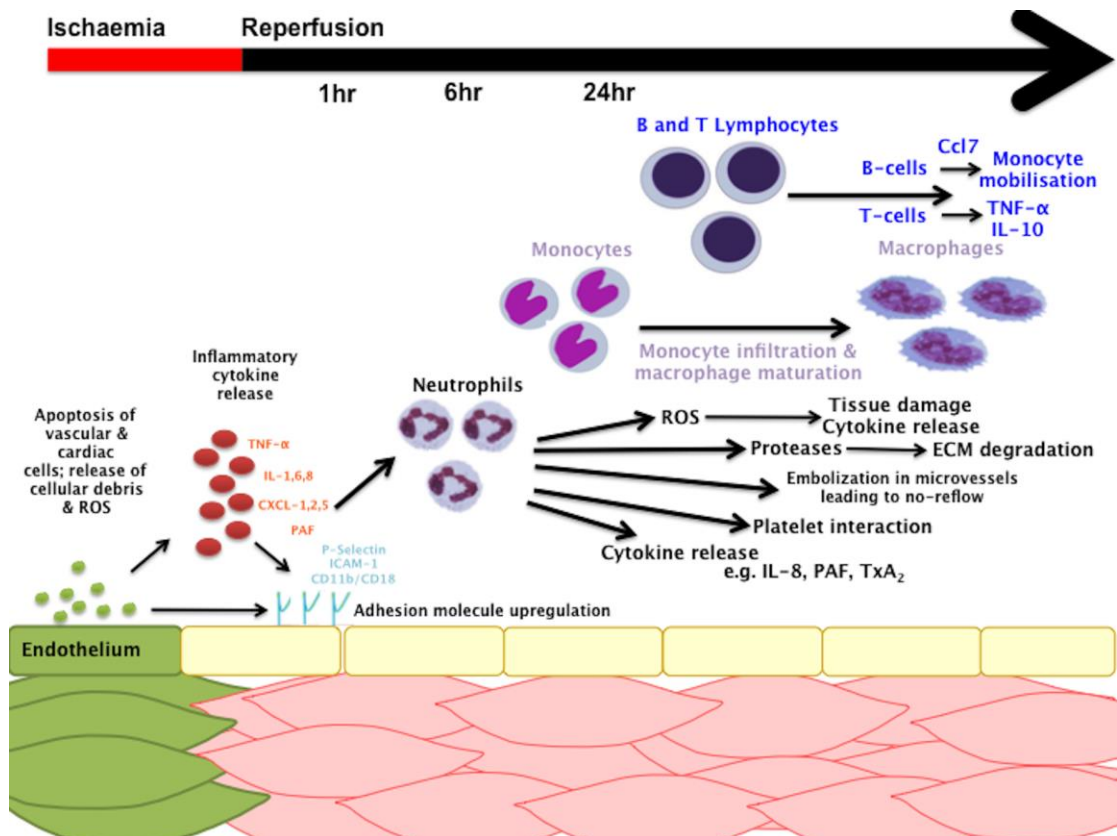
Animal models show that neutrophil accumulation in the reperfused area at risk is most rapid during the first hour with landmark data (Zhao et al., 2000a) demonstrating an abundance of neutrophils accumulating in the myocardium, localized in the intravascular space during the first six hours of reperfusion, followed thereafter by a shift into the parenchyma as they migrate transendothelially over the ensuing 24 hours. This process is facilitated by up-regulated cell-adhesion molecules P-selectin (Davenpeck et al., 1994), CD18/CD11 (Entman et al., 1990), and ICAM-1 (Kukielka et al., 1993; Youker et

al., 1994), which aid the migration of neutrophils into the myocardial tissue. The upregulation of ICAM-1 on cardiomyocytes is stimulated by cytokine release, and activated neutrophils adhere to cytokine (IL-1, IL-6, TNF $\alpha$ ) stimulated cardiomyocytes by a CD11b/CD18-ICAM-1 dependent mechanism (Entman et al., 1990).

Neutrophils can contribute to the cell death in ischaemic-reperfused myocardium directly or indirectly by a number of mechanisms: (1) the release of ROS during the early interactions with the coronary endothelium which precedes significant transendothelial migration (Albertine et al., 1994); (2) the release of proteases such as elastase and collagenase (Weiss, 1989); (3) embolization in microvessels leading to no-reflow and secondary ischaemia (Mehta et al., 1988; Siminiak et al., 1995); (4) damage to the coronary vascular endothelium and upregulation of adhesion molecules implicated in amplification of neutrophil adhesion and emigration (Jordan et al., 1999); (5) release of lipoxygenase products such as leukotriene B<sub>4</sub> (LTB<sub>4</sub>) which acts synergistically with thromboxane A<sub>2</sub> causing intense vasoconstriction (Nichols et al., 1988) and also acts as a potent chemoattractant (Perlman et al., 1989); (6) promotion of the interaction with platelets that may further potentiate ischaemia-reperfusion injury (Alloatti et al., 1992); (7) extension of infarct size and the degree of apoptosis during later phases of reperfusion (Zhao et al., 2001; Zhao et al., 2000b).

The wave of neutrophil infiltration is followed by the mobilization and recruitment of monocytes. Recent studies have shed light on the mechanisms by which monocytes are recruited to the heart and the life cycle of the recruited

monocytes in the setting of AMI. These studies have suggested that Ly-6C<sup>hi</sup> (mouse equivalent of human inflammatory 'CD14<sup>hi</sup>CD16<sup>-</sup>' monocytes), and Ly-6C<sup>lo</sup> (mouse equivalent of human resident 'CD14<sup>lo</sup>CD16<sup>+</sup>' monocytes) monocytes have pathogenic and protective roles, respectively, in cardiac remodeling and preservation of heart function (Nahrendorf et al., 2007) (Leuschner et al., 2012).



**Figure 1.6. Temporal events of the inflammatory response following myocardial infarction**

The inflammatory response following reperfusion therapy for myocardial infarction is characterized by the sequential infiltration of the injured myocardium with neutrophils, monocytes and lymphocytes. This is a dynamic process: recruitment or proliferation of each cell type is followed by activation. Various cell populations have distinct but overlapping functions.

After migration of monocytes into the myocardial tissue, they become active macrophages, releasing a variety of inflammatory substances, cytokines, and growth factors (Wan et al., 1997). Mast cells are also actively implicated in the post-MI inflammatory response and cardiac remodeling as they are stimulated by



the complement 5a (C5a)-adenosine-reactive oxygen complex, a necessary element for the development of the myocardial stunning process, part of I/R injury. In fact, by degradation, they may release vascular endothelium growing factors, fibroblast growing factors, and histamine, all promoting elements of myocardial fibrosis (Serini and Gabbiani, 1999; Kereiakes 2003).

It had been felt that that adaptive immune system played little role in the inflammatory contribution to I/R injury however recent studies have suggested a key involvement for lymphocytes in reperfusion injury. T lymphocytes have been demonstrated to be pathogenic during myocardial I/R injury. Yang et al (Yang et al., 2006b) evaluated myocardial infarct size in wild type control and RAG1 knockout (KO) mice (RAG1 plays an important role in VDJ recombinase, a complex of enzymes that work to join gene segments of B and T cell receptor genes, therefore RAG1 KO mice contain no mature B and T lymphocytes (Mombaerts et al., 1992) following 45 minutes of left anterior descending (LAD) coronary artery occlusion. RAG1 KO mice had significantly smaller infarct sizes compared to that of control mice. After reconstitution of RAG1 KO mice by adoptive transfer with CD4<sup>+</sup> T cells, the infarct size of the reconstituted RAG1 KO mice was significantly greater than that of the RAG 1 KO. They also examined T-cell depletion and its effect on myocardial infarct size. CD4<sup>+</sup> depleted mice, but not CD8<sup>+</sup> depleted mice had a significantly decreased infarct size compared to control mice again implicating CD4<sup>+</sup> T cells in a deleterious role in I/R injury (Yang et al., 2006b). Further support for CD4<sup>+</sup> T cells comes from data demonstrating a cardioprotective effect of interleukin-10, a cytokine that suppresses interferon- $\gamma$  secreting CD4<sup>+</sup> T cell levels 4 days after AMI in a mouse

model (Burchfield et al., 2008). Little data exists assessing the T cell contribution to I/R injury in humans however a recent study looking at circulating T cell populations following primary PCI for STEMI identified a depletion of CCR7<sup>+</sup>/CD4<sup>+</sup> T cells from peripheral blood during the first 30 min of reperfusion following primary PCI (Hoffmann et al., 2012). CCR7 has been identified as a key regulator of T-cell trafficking to secondary lymphoid organs and can also be involved in the abnormal recruitment of T cells from the peripheral blood to sites of acute inflammation (Forster et al., 2008). Therefore, it is conceivable that T cells could be migrating to infarcted myocardium via a CCR7 dependent mechanism. Although it should be noted that despite the 'disappearance' of T-cells from the peripheral blood it cannot be concluded that the remaining cells have entered the myocardium (Hoffmann et al., 2012).

Increasing evidence is emerging suggesting a role for systemic B cells in the immune responses leading to tissue damage after AMI (Chen et al., 2009; Zouggari et al., 2013). A recent study using mouse models demonstrated that after AMI (in vivo ligation of the left coronary artery) in mice, mature B lymphocytes selectively produce Ccl7 and induce Ly6C<sup>hi</sup> monocyte mobilization and recruitment to the heart, leading to enhanced tissue injury and deterioration of myocardial function (Zouggari et al., 2013). Reversing this effect using either models of Baff receptor deficiency (Baff signaling/receptor is required for the maintenance of mature B lymphocytes) or antibodies against CD20<sup>+</sup> or Baff resulting in depletion of mature B lymphocytes impeded Ccl7 production and monocyte mobilization, limited myocardial injury and improved heart function. These effects were also recapitulated in mice with B cell-selective Ccl7 deficiency.

In separate experiments it has been shown that high circulating concentrations of Ccl7 and Baff in patients with AMI predict increased risk of death or recurrent myocardial infarction (Zouggari et al., 2013). This suggests an interaction between mature B lymphocytes and monocytes after AMI that contributes significantly to I/R injury. Further study is required to assess the exact contribution of both B and T lymphocytes in I/R injury.

Various approaches have been used to inhibit the inflammatory component of I/R injury, ranging from strategies that specifically target the cellular components of inflammation by physically or chemically removing neutrophils from the systemic or coronary circulation, and systemic anti-inflammatory agents. These studies have shown reductions in infarct size of up to 50%. Specific interventions targeting cellular recruitment include leukocyte depleted blood (Litt et al., 1989); antibodies against the cell-adhesion molecules P-selectin (Hayward et al., 1999), CD11 and CD18 (Ma et al., 1991) and ICAM-1 (Zhao et al., 1997) and pharmacologic inhibitors of complement activation (Vakeva et al., 1998). However, despite these promising results a number of experimental studies have also reported a lack of reduction of infarct size with systemic anti-inflammatory therapy (e.g. using non-steroidal anti-inflammatory drugs such as ibuprofen or indomethacin) in animal models (Allan et al., 1985; Reimer et al., 1985). In the study by Reimer et al, (Reimer et al., 1985) in a canine model with 3 hours of coronary artery occlusion followed by 3 days of reperfusion ibuprofen treatment did not reduce infarct size. Anti-inflammatory effects have been proposed to underlie the beneficial effects of adenosine in mice following its administration with experiments using intravenous, intra-coronary or topical administration

yielding positive results (Kaminski and Proctor, 1989; Babbitt et al., 1989). However using the intravenous approach adenosine has also been shown to be ineffective in a separate study, with the short half-life being proposed to be responsible for this lack of effect (Vander Heide and Reimer, 1996).

Based on these promising pre-clinical results several agents have been proposed as potential therapies for translation into human subjects including adenosine however caution is warranted. The 'prototype' infarct size reduction study tested high-dose methylprednisolone (eight 30-mg/kg doses every 6 hours) as an anti-inflammatory treatment in patients with AMI (Roberts et al., 1976), this showed an augmentation of infarct size (measured enzymatically), accentuation of malignant arrhythmias and in some cases ventricular aneurysm formation and rupture. This trial highlighted the double-edged sword of anti-inflammatory therapy, in that long-term treatment can attenuate the healing process as well as the acute inflammatory response in post-myocardial infarction. Studies assessing steroid therapy in AMI have subsequently not shown such damaging effects but have also not shown benefit (Heikkila and Nieminen, 1978; Madias and Hood, 1982). Many other studies aimed at reducing the inflammatory response post AMI have also been negative, either with therapies aimed at specific targets; antibodies against CD18 (Baran et al., 2001b; Faxon et al., 2002), antibodies against P-selectin (Alfonso and Angiolillo, 2013), Anti-C5 antibody (pexelizumab) (Mahaffey et al 2003; Armstrong et al., 2007) or systemic therapies; NSAIDS (Gibson et al., 2009), or either intravenous or intracoronary adenosine (Ross et al., 2005; Desmet et al., 2011).

In concert, these mechanisms produce irreversible damage to cardiomyocytes that are severely ischaemic but still salvageable at the time of reperfusion. Consequently, these pathways have been the therapeutic targets in experimental and clinical studies. Importantly, these interventions do seem to translate better than the pharmacological interventions with recent trials of both remote conditioning (Botker et al., 2010; Sloth et al., 2014) and post conditioning (Thuny et al., 2012) demonstrating benefits. Additional endogenous pathways, which may be potentially cardioprotective, have also been identified which are discussed below.

#### **1.3.6 Survival Kinase pathways**

The reperfusion injury salvage kinases (RISK) are a group of proteins that are activated during myocardial reperfusion, conferring cardioprotection (Hausenloy and Yellon, 2004). They emerged as a concept in the late 1990s with the recognition that apoptotic cell death contributed to lethal reperfusion injury (Gottlieb et al., 1994; Freude et al., 2000), and the knowledge that certain pro-survival anti-apoptotic protein kinases, such as Akt and extraregulated kinase 1 and 2 (Erk1/2) existed, which when specifically activated at the time of myocardial reperfusion conferred powerful cardioprotection (Yellon and Baxter, 1999; Hausenloy and Yellon, 2004). Subsequent study has further identified this pathway and it is now known to include other cardioprotective reperfusion salvage kinases such as protein kinase C (PKC) (primarily the PKC-ε isoform) (Wolfrum et al., 2002), protein kinase G (PKG) (Han et al., 2001), p70s6K (Tsang et al., 2004), and glycogen synthase kinase-3β (GSK-3β) (Tong et al., 2002).

There is extensive preclinical evidence that activation of the RISK pathway by either pharmacological agents such as atorvastatin (Bell and Yellon, 2003), erythropoietin (Bullard et al., 2005), and atrial natriuretic peptide (ANP) (Yang et al., 2006a) or by mechanical interventions such as postconditioning (Hausenloy et al., 2005) reduces myocardial infarct size by up to 50%. It is believed that activation of the RISK pathway confers cardioprotection by blocking calcium overload (Abdallah et al., 2006), upregulating anti-apoptotic pathways (Yellon and Baxter, 1999) and via inhibition of mPTP opening (Davidson et al., 2006), mechanisms all suggesting that the different proposed mechanisms involved in cardioprotection are interconnected, sharing common final pathways.

However the RISK pathway is not critical to all cardioprotective agents. TNF- $\alpha$  has been shown to mimic postconditioning in isolated murine hearts in the absence of Akt activation, with its protective effect unaffected by the PI3K inhibitor, Wortmannin (Lacerda et al., 2009). Similarly ischaemic postconditioning in pigs in vivo (Skyschally et al., 2009) has also been shown to be independent of RISK activation. Taken together, these data support the existence of an alternative pro-survival signal transduction pathway for protecting the ischaemic myocardium against lethal myocardial I/R injury. Mechanistic studies have demonstrated that TNF- $\alpha$  initiates the activation of a novel and alternative protective path which has been termed the Survivor Activating Factor Enhancement (SAFE) pathway (Lecour, 2009) which involves the activation of Janus kinase (JAK), signal transducer and activator of transcription 3 (STAT-3), rather than the RISK pathway (Lecour et al., 2005; Suleman et al., 2008). Further work is needed to elucidate the relationship

between these two signaling pathways with studies ongoing to investigate the interplay between the pathways. It has been suggested that both the RISK and SAFE pathways seem to converge on the mitochondria and the mPTP, which is believed to be the target or end effector for the protection (Heusch et al., 2008).

#### **1.4 Clinical translation of cardioprotective strategies**

Whereas many strategies have been shown to be effective in reducing I/R injury in pre-clinical models, the translation of these approaches into the clinical setting has been disappointing (Yellon and Hausenloy, 2007). Most strategies have attempted to address individual mechanisms underlying I/R injury such as ROS or inflammation. Examples of this include the targeting of ROS with free-radical scavengers or anti-oxidants (Flaherty et al., 1994; EMIP-FR Group, 2000), trials of inhibition of the Na<sup>+</sup>-H<sup>+</sup> exchanger (Zeymer et al., 2001), and measures aimed at reducing inflammatory damage induced by neutrophils (Baran et al., 2001a; Mertens et al., 2006), all of which have been unsuccessful. Additionally clinical studies targeting the generalised inflammatory components of myocardial I/R injury with anti-inflammatory agents such as adenosine have failed to demonstrate any impact on clinical outcomes in reperfused-STEMI patients (Ross et al., 2005; Armstrong et al., 2007). Trials targeting metabolic function such as glucose-insulin-potassium infusions (Mehta et al., 2005) or magnesium therapy (Woods et al 1992; ISIS-4 Study Group, 1995) also have generated inconclusive results. The reasons for the failure in translation are multiple but key factors are thought to include:-

- 1) Several of the failed interventions, including anti-oxidants, calcium-channel antagonists, adenosine, and erythropoietin had not shown conclusive cardioprotection in the pre-clinical animal studies, which may in part explain why they failed in the clinical setting
- 2) Many of the study interventions were designed to target only one component of myocardial I/R injury such as ROS, calcium channel accumulation, apoptosis, and inflammation and not effect either all pathways or a final common one such as the mPTP.
- 3) The design of the clinical cardioprotection study: it is essential that the design of the clinical study take into consideration the findings of previously published pre-clinical and clinical studies. This includes factors such as dose, mode of delivery and measurement of effect.

Recently, improved understanding of the pathophysiological mechanisms underlying I/R injury such as identification of the mPTP have resulted in the design of therapies no longer directed at individual pathways. This coupled with excellent pre-clinical evidence and improved trial design has resulted in the identification of several promising mechanical (ischaemic post-conditioning (Staat et al., 2005) and remote ischaemic pre-conditioning (Botker et al., 2010) (Sloth et al., 2014), and pharmacological (cyclosporine (Piot et al., 2008), exenatide (Lonborg et al., 2012b)) therapeutic strategies, for preventing myocardial I/R injury.



### **1.4.1 Mechanical Therapies**

In 2003, Zhao and coworkers demonstrated that short periods of ischaemia and reperfusion applied immediately after the reopening of an occluded LAD could reduce I/R injury and infarct size in open chest anaesthetized dogs, a concept referred to as ischaemic postconditioning (Zhao et al., 2003). In animal studies, ischaemic postconditioning involves all major contributors to reperfusion injury including the RISK pathway and the mPTP. This therapeutic approach was rapidly translated into the clinical setting of primary PCI in 2005 (Staat et al., 2005) where the cumulative creatine kinase (CK) release measured over 72 hours following reperfusion was reduced by a protocol of four cycles of 1 min angioplasty balloon inflation followed by 1 min deflation after reopening of the infarct related artery. The cumulative 72-hour CK release vs. the area at risk, as determined using ventriculography before reperfusion from the abnormally contracting myocardium, was also reduced. However the results of studies performed since have been inconsistent, with debate ongoing as to the effectiveness of this therapy (Freixa et al., 2012; Hahn et al., 2013).

**Remote Ischaemic conditioning:** The phenomenon of remote ischaemic conditioning (RIC) allows the therapeutic intervention (transient episodes of ischaemia and reperfusion) to be applied to an organ or tissue distant from the heart, thereby facilitating its clinical application. In 1993, it was first demonstrated in a canine model that applying four 5-minute cycles of occlusion and reflow to the circumflex coronary artery could reduce infarct size generated by a sustained occlusion in the LAD (Przyklenk et al., 1993) This study

demonstrated for the first time that cardioprotection could be transferred from one region of the heart to another. This concept was quickly extended beyond the heart with the demonstration that the myocardium could be protected against MI by applying the preconditioning stimulus to either the kidney or intestine (Gho et al., 1996), confirming that the heart could be protected at a distance.

Two independent clinical studies have been performed investigating the effect of RIC in STEMI patients undergoing primary PCI. In the 1<sup>st</sup> study (Rentoukas et al., 2010), 96 STEMI patients were randomised at the hospital to receive control, RIC (3 cycles of blood pressure cuff placed on the upper limb to 20 mm Hg above systolic arterial pressure for 4 min and deflating the cuff for 4 minutes), or RIC + morphine (5 mg intravenous infusion initiated 5 minutes before reperfusion). In those STEMI patients who received RIC alone, there was a non-significant trend to benefit when compared to control patients. In the 2<sup>nd</sup> larger study (Botker et al., 2010) STEMI patients were randomised in the ambulance to receive control (a deflated cuff placed on the upper arm for 40 minutes) or RIC (four 5-minute inflations to 200 mmHg and deflations to 0 mmHg of upper arm cuff). Patients were randomised in the ambulance prior to coronary angiography and the primary endpoint was myocardial salvage index at 30 days post primary PCI. Although 333 patients were randomised, only 142 patients were analysed for the primary endpoint of myocardial salvage index as others were unsuitable. Those patients randomised to RIC had a greater myocardial salvage index when compared to control (0.75 vs. 0.55,  $p=0.0333$ ), although there was no significant difference in final infarct size at 30 days (4% vs. 7% of LV,  $p=0.1$ ). However, in those patients with greater AAR (LAD infarcts and complete occlusion in the

infarct-related coronary artery (TIMI 0 flow)) there was a greater reduction in final infarct size at 30 days in those patient treated with RIC when compared to control (8% vs. 16% of LV with LAD STEMI,  $p=0.01$  and 9% vs. 13% of LV with TIMI 0,  $p=0.06$ ), despite there being little difference in the myocardial salvage index. However, it is important to note there were no significant differences in secondary outcomes including peak troponin T, ST-segment resolution and LVEF at 30 days (Botker et al., 2010). The mechanisms underlying RIC cardioprotection remain unclear but have been attributed to neurohormonal pathways conveying the cardioprotective signal from the limb to the heart (Hausenloy and Yellon, 2008)

#### **1.4.2 Pharmacological Therapies**

The first pharmacological agent to show promise in translating into patients undergoing primary PCI for STEMI was cyclosporine. The rationale behind its use is that cyclosporine is a potent inhibitor of mPTP opening (Nazareth et al., 1991a). This inhibition of opening is via its binding to the cyc-D located in the mitochondrial matrix (Woodfield et al., 1998). In a proof of concept trial cyclosporine, was injected intravenously at a dose of 2.5mg/kg in 58 patients (28 placebo, 30 cyclosporine) before primary PCI for AMI, and was shown to significantly reduce infarct size as assessed by CK profile over 72 hours. No difference was seen in the troponin T profile over the same time-period. In addition, in a sub-group of 27 patients it was demonstrated, by CMR imaging 5 days post-primary PCI, that cyclosporine treatment was associated with a reduced area of hyper-enhancement (infarcted tissue) (Piot et al., 2008). An

additional follow-up sub-study of the patients who underwent CMR showed a persistent reduction in infarct size at 6 months in the cyclosporine group compared with the control group of patients ( $29 \pm 15$  g vs.  $38 \pm 14$  g;  $p = 0.04$ ). There was also a significant reduction of LV end-systolic volume (and a trend for LV end-diastolic volume;  $p = 0.07$ ) in the cyclosporine group compared with the control group, both at 5 days and 6 months after infarction (Mewton et al., 2010).

The other promising pharmacological therapy is exenatide, a synthetic analogue of exendin-4, also known as “lizard spit” which is found in the salivary secretions of the Gila monster, *Heloderma suspectum*. Importantly this 39-residue reptilian peptide is pharmacologically similar to mammalian glucagon-like peptide (GLP)-1 (Treiman et al., 2010). Receptors for GLP-1 have been found in the heart and in rat (Sonne et al., 2008) and pig (Timmers et al., 2009), GLP-1 or its analogues protect against reperfusion injury-induced cell death in these species. This has subsequently been shown in 2 studies to have a cardioprotective effect in clinical studies of patients undergoing primary PCI for STEMI. Lønborg and co-workers showed that a 6-hour continuous exenatide infusion (at a rate of 26 mL/h ( $0.043 \mu\text{g}/\text{min}$ ), to achieve a plasma concentration of 0.03 and 0.3 nmol/L) during reperfusion therapy in patients presenting with STEMI resulted in an improvement in myocardial salvage in a group of 105 patients with reduction of infarct size in a subgroup with short systemic delay ( $\leq 132$  minutes) (Lønborg et al., 2012b). More recently a smaller study (Woo et al., 2013) assessing 58 patients with STEMI who received either exenatide (10  $\mu\text{g}$  subcutaneous and 10  $\mu\text{g}$  intravenous bolus 5 minutes before reperfusion with further 10  $\mu\text{g}$  twice daily doses for 2 days) or placebo (received an equivalent volume of normal saline

with the same treatment regimen) showed a reduction in infarct size (assessed by CK-MB and troponin I) in the exenatide group compared to placebo. There was also a significant reduction in CMR assessed infarct size in the exenatide group ( $12.8 \pm 11.7$  versus  $26.4 \pm 11.6$  g;  $P < 0.01$ ). However, patients with left ventricular systolic dysfunction were excluded from the study and the use of exenatide was associated with significantly higher rates of nausea (56%), vomiting (33%) and loss of appetite (28%) with 1 patient experiencing symptomatic hypoglycemia requiring glucose infusion to complete the study (Woo et al., 2013).

Despite the potential of these promising strategies limitations exist either with regard to route of administration, potential side-effects and complications and inconsistent results in clinical trials. The intravenous route of administration utilized with exenatide/cyclosporine will possibly result in a delay in reperfusion. In trials of both agents the intervention was administered following confirmation of TIMI flow  $\leq 1$ . Additionally the intra-venous administration may result in greater side-effects due to systemic drug delivery. For example with exenatide (Woo et al., 2013) higher rates of adverse effects in the exenatide group have been shown compared to control (although this did not result in subject withdrawal). Similarly mechanical therapies come with additional risks such as ischaemic postconditioning where consecutive occlusions using a balloon catheter after the opening of the culprit vessel carries a potential risk of damaging the vessel or inducing peripheral embolization, similar risks exist with RIC if performed locally in a non-culprit coronary vessel. For both mechanical therapies currently clinical benefit is debated with inconclusive results in current

attempts at translation with large studies ongoing assessing the effectiveness of both techniques.

A new potential solution to the problem of I/R injury may exist in the form of inorganic nitrite ( $\text{NO}_2^-$ ). Over the last decade evidence has accumulated supporting the view that nitrite, which is abundant in blood and tissues (Bryan et al., 2005; Webb et al., 2008; Li et al., 2008; Feelisch et al., 2008), represents a significant stable intravascular endocrine reservoir and tissue storage form of NO that exerts a number of beneficial effects (Lundberg JO and Weitzberg E, 2008). Importantly, a deficit of bioavailable NO contributes to the pathological events associated with both I/R injury and the long-term outcomes post-PCI. Therefore strategies that might improve NO delivery during PCI and post PCI may offer therapeutic benefit and this might be provided in the form of inorganic nitrite.

## **1.5 NO and the cardiovascular system**

### **1.5.1 Discovery**

Since its discovery 25 years ago, NO has emerged as an important signaling molecule in a myriad of pathways, involving the nervous, immune, and most relevant to this thesis, the cardiovascular system. NO was initially discovered in an attempt to reconcile the apparent conflict in the actions of acetylcholine (ACh) in vivo and in vitro. Although ACh is normally a potent vasodilator in vivo, it was often found to have little vasorelaxant effect in isolated vessel preparations. However it was Furchgott and Zawadzki in 1980, who discovered that during

preparations of vessels for in vitro studies, accidental denuding of the endothelial layer caused loss of responsiveness to ACh, yielding the conflicting results (Furchgott and Zawadzki, 1980). In this paper Furchgott and Zawadzki suggested that the endothelial cells were releasing a substance that was crucial for the ACh-induced vasodilation. The term endothelial-derived relaxation factor (EDRF) was used to describe this unknown entity (Furchgott and Zawadzki, 1980). Eventually, NO was identified as the EDRF responsible for mediating vasodilation in response to ACh (Palmer et al., 1987). Upon its synthesis, endothelial NO was shown to be a potent stimulator of soluble guanylate cyclase (sGC) to form cyclic guanylate monophosphate (cGMP) and cause relaxation of vascular smooth muscle cells (VSMC) (Arnold et al 1977; Ignarro et al., 1981). These investigations into the role of NO as a signaling molecule in the cardiovascular system earned Drs Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad a Nobel Prize in Physiology and Medicine in 1998 (Furchgott, 1993; Ignarro et al., 1999).

### **1.5.2 Conventional pathway of NO generation**

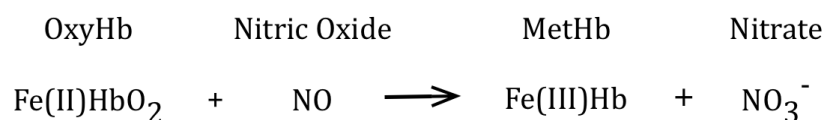
Under normal conditions, within the cardiovascular system, basal endogenously generated NO, produced from the amino acid L-arginine by a family of enzymes known as NOSs, plays an essential role in maintaining homeostasis and, of particular relevance to this thesis, in sustaining normal healthy cardiac function, perfusion and cardioprotection (Bredt, 2003; Mount et al., 2007). There are three isoforms of NOS that have been characterized; the endothelial isoform (eNOS, also known as NOSIII), the neuronal isoform (nNOS, also known as NOSII) and the inducible isoform (iNOS, or NOSI)(Bredt and Snyder, 1990; Stuehr, 1999). It is

eNOS however, that is of particular importance for cardiovascular physiology and pathophysiology as it maintains basal vascular NO production that exerts a range of beneficial effects (anti-platelet, anti-inflammatory, vasodilatation) that sustain cardiovascular health (Moncada and Higgs, 1993). eNOS is constitutively expressed and regulated by calcium, calmodulin and by post-translational modification (Michel et al., 1997).

The NO synthases catalyze the five-electron oxidation of L-arginine to NO and L-citrulline using NADPH as the electron source (Figure 1.7). This reaction is dependent on oxygen and the enzymes require several co-factors for proper function, including BH<sub>4</sub>, nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide, and flavin mononucleotide (Moncada and Higgs, 1993) (Figure 1.7) Therefore, NO synthesis is significantly influenced by the presence of these co-factors, as well as substrate and oxygen availability (Michel and Feron, 1997).

NO is a free radical with a biological half-life in the millisecond range (Borland, 1991; Liu et al., 1998). This short half-life makes its detection in vivo difficult and therefore the end products of NO reaction pathways are usually measured to quantify NO production and metabolism. As with many substances NO has a very rapid metabolism pathway. The predominant pathway is through a reaction first described nearly 150 years ago in 1865 by Hermann (as cited by Gladwin et al 2005) where binding with oxyhaemoglobin (oxyHb) generates methaemoglobin (metHb) and nitrate (NO<sub>3</sub><sup>-</sup>) (Equation 1.1).



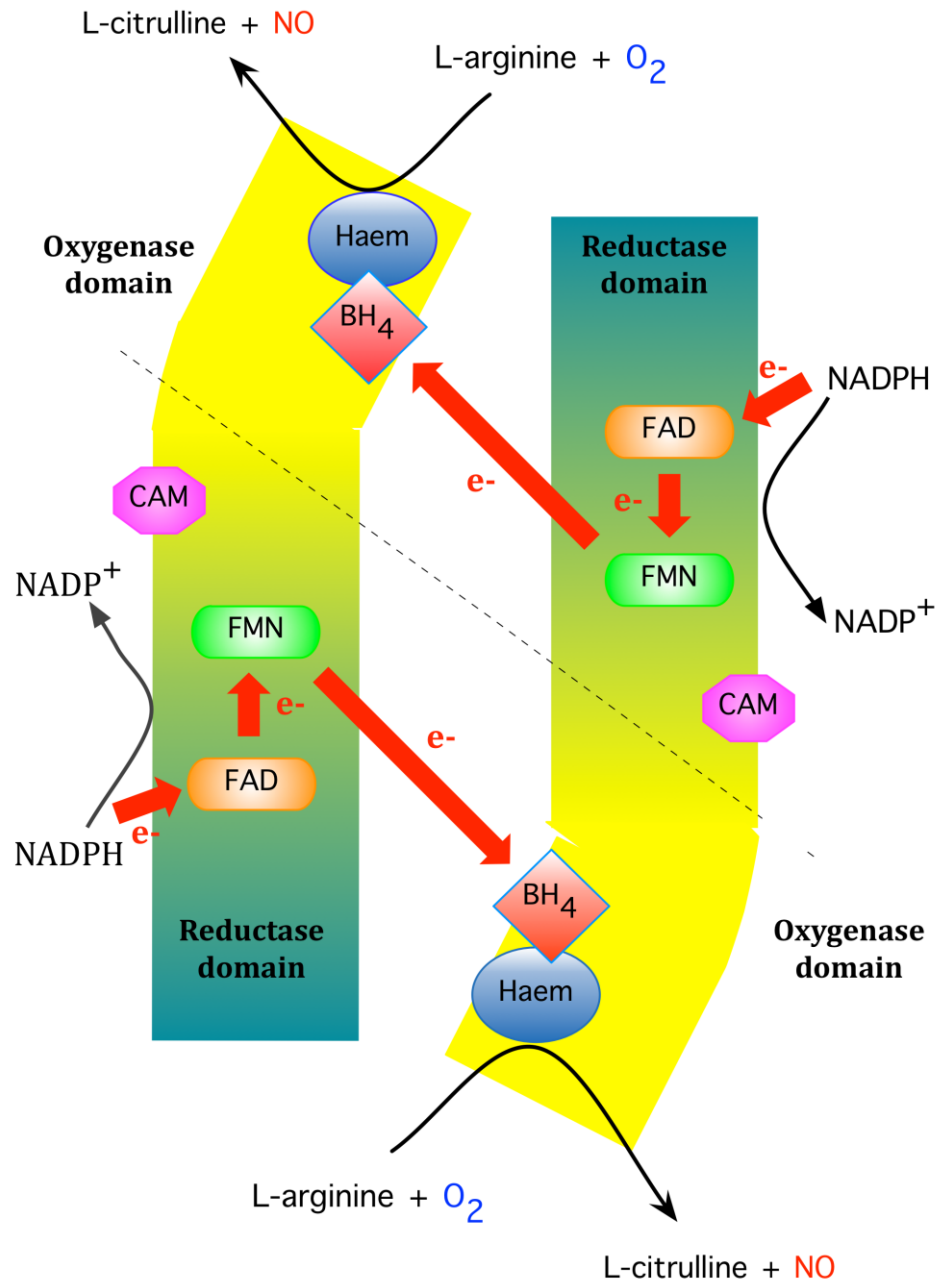


**Equation 1.1. Oxidative termination of NO activity with Hb (Hb=haemoglobin, NO= nitric oxide)**

This incredibly fast and rapid reaction ( $6\text{-}8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ) represents the major pathway for the destruction of NO (Feelisch and Noack, 1987; Herold et al., 2001).

Additionally NO rapidly reacts with species that possess unpaired electrons (e-) such as  $\text{O}_2$ , which is the main oxidative mechanism to form nitrate in aqueous solutions and radicals such as  $\text{O}_2$ . In pure aqueous solutions, this oxidation of NO occurs slowly and the primary product is nitrite (Ignarro et al., 1993), however, with the addition of oxyhaemoproteins, such as oxyHb, then the predominant product is  $\text{NO}_3^-$  (Ignarro et al., 1993) and thus nitrate is the predominant oxidative metabolite of NO in biological systems.

NO reacts with a variety of metals to form nitrosyl complexes, most commonly with iron (Fe) but also copper (Cu) (Fukuto et al., 2000). One classic Fe nitrosyl reaction that occurs is the activation of haem containing protein sGc. Other haem-containing proteins that have important interactions with NO, include cytochrome P450 and Hb itself (Fukuto et al., 2000).



**Figure 1.7. Coupled Dimeric eNOS and co-factors.**

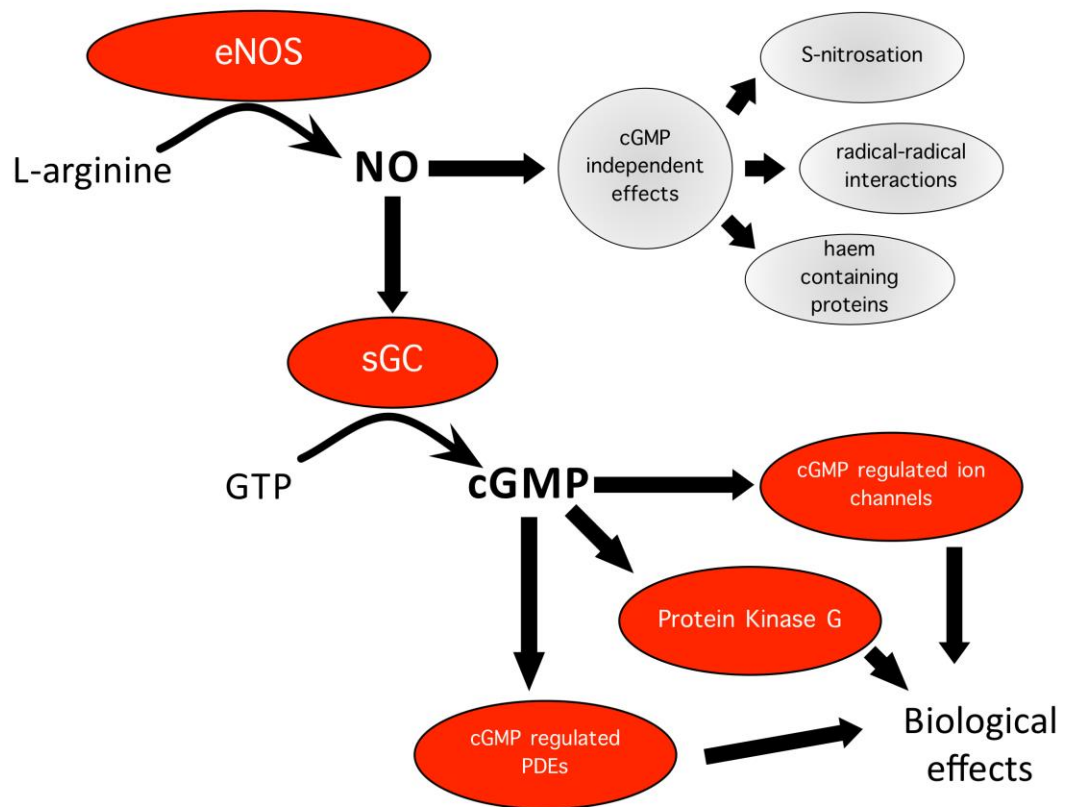
Diagrammatic representation of the domains and cofactors required for coupled eNOS synthesis of NO. (CAM=calmodulin; eNOS= endothelial nitric oxide synthase; FAD=flavin adenine dinucleotide; Fe=iron; FMN=flavin mononucleotide; BH<sub>4</sub>=tetrahydrobiopterin; NADPH=nicotinamide adenine dinucleotide phosphate). The red arrows depict electron flow eNOS. Electron flow starts from NADPH to flavins FAD and FMN of the reductase domain, which delivers the electrons to the Fe of the haem (oxygenase domain) and to the BH<sub>3</sub><sup>•</sup> radical generated as an intermediate in the catalytic cycle. BH<sub>4</sub> is essential to donate an electron and proton to versatile intermediates in the reaction cycle of L-arginine/O<sub>2</sub> to L-citrullin/NO. Calmodulin (CAM) controls electron flow in eNOS.

The majority of the beneficial effects of NO in the cardiovascular system are mediated by cGMP (for review see Murad, 2006). NO diffuses through the lipid phase of cell membranes and into the cytosol where it meets its target protein, the enzyme sGC, binding to its haem group and causes a 100-200-fold increase in its activity (Ignarro et al., 1982; Stone and Marletta, 1996; Friebe and Koesling, 2003). Activation of sGC converts the nucleotide guanosine triphosphate (GTP) into cGMP (Waldman and Murad, 1988; Furchgott and Jothianandan, 1991). cGMP itself has a molecular target identified nearly 40 years ago as a cGMP-dependent protein kinase (now known as PKG) (Greengard, 1975). Activation of PKG leads to a series of signaling events with diverse consequences, including vascular smooth muscle relaxation (Moncada and Higgs 1993; Schmidt and Walter, 1994; Tang et al., 2003).

The NO-induced cGMP signal also has effects on other intracellular effector molecules; cGMP-regulated phosphodiesterases (PDEs), and cGMP-gated ion channels. In the cardiovascular system, the primary cGMP-PDEs with known activity are PDE1, PDE2, and PDE5 (of 11 so far discovered) (Kass et al., 2007) (Figure 1.8).

NO has cGMP independent effects; it exerts widespread signaling through the post-translational modifications of proteins and small molecules via S-nitrosation of sulfhydryl groups (Gow et al., 1997), which has been demonstrated in well over 100 proteins in multiple cells and tissues, including the heart (Stamler, 1995; Gow et al., 2002; Hess et al., 2005). Various proteins involved in the regulation of myocardial contractility are modified by protein S-nitrosation (Xu et

al., 1998). For example in cardiac muscle, NO S-nitrosylates the ryanodine receptor on the sarcolemmal membrane, which is required for its normal activity (Xu et al., 1998). NO has also been shown to regulate gene expression and messenger ribonucleic acid (mRNA) transcription via binding to Fe response elements (Pantopoulos and Hentze, 1995; Khan et al., 1996).



**Figure 1.8 NO/cGMP signaling cascade**

NO produced by eNOS activates sGC and leads to increased synthesis of cGMP. This intracellular messenger in turn modulates the activity of cGMP-dependent protein kinase G, cGMP-gated ion channels and cGMP-regulated phosphodiesterases. These effectors are involved in the regulation of multiple physiological functions in the cardiovascular system.

### **1.5.3 NO and I/R injury**

The cardioprotective effects of NO relate to a number of actions including its potent vasodilator action in the ischaemic myocardium (Johnson G III et al., 1991), which allows for essential perfusion of injured tissue. In addition NO exerts its anti-inflammatory effects with respect to inhibition of leukocyte recruitment (Kubes et al., 1991; Ahluwalia et al., 2004) which reverses the inflammatory cell driven injury to the endothelium and perivascular myocardium that plays a major role in determining infarct size post myocardial I/R injury (dPalazzo et al., 1998). NO also exerts anti-platelet effects, inhibiting platelet adhesion (Queen et al., 2000), aggregation (Radomski et al., 1990), recruitment (Freedman et al., 1997), and the formation of leucocyte-platelet aggregates (Chung et al., 2004). These anti-platelet effects, combined with the anti-leukocyte actions of NO helps to attenuate capillary plugging (Jones and Bolli, 2006). Additionally, in the long term following acute myocardial I/R injury it is thought that deficient NO generation contributes to delay in re-endothelialisation of blood vessels following primary PCI, enhances cardiomyocyte death and apoptosis during I/R injury, contributes to cardiomyocyte hypertrophy post-infarct and decreases the impact of strategies that might facilitate new cardiomyocyte generation (Razavi et al., 2005; Dorn, 2009). Importantly and crucially for cardioprotection, NO prevents the opening of the mPTP (Brookes et al 2000; Heusch et al., 2008) which as discussed earlier is a critical and final common step in reperfusion injury (Griffiths and Halestrap, 1995) (section 1.3.5).

During an I/R insult the activity of NO is compromised. The ability of eNOS to

make NO is severely attenuated as a result of inadequate delivery of oxygen and co-factors (Giraldez et al., 1997). This was first shown in isolated rat hearts where eNOS activity (assayed by L-[<sup>14</sup>C]arginine to L-[<sup>14</sup>C]citrulline conversion) although shown to be preserved after 30 minutes of ischemia with a value of  $1.1 \pm 0.1$  pmol of L-citrulline/min x mg of protein, was decreased by 77% after 60 minutes and became nearly undetectable after 120 min. Reperfusion resulted in only a partial restoration of activity. Haemodynamic studies showed that the onset of impaired vascular reactivity paralleled the loss of functional eNOS (Giraldez et al., 1997). In addition, reperfusion generates ROS particularly generation of  $O_2^-$ , which binds avidly to NO generating ONOO<sup>-</sup>. This scavenging further reduces the bioavailability of NO, removing its cardioprotective effects (Bolli, 2001) (Moens et al., 2005). In addition, ONOO<sup>-</sup> is a short-lived oxidant, which is thought to be significantly more reactive and damaging than its precursors and is thus more toxic to cells than H<sub>2</sub>O<sub>2</sub>,  $O_2^-$ , or NO alone (Murphy et al., 1998). Together, such pre-clinical observations are consistent with a protective role for NO (Jones and Bolli, 2006) and support the concept of pharmacotherapy focused on replacing the protective NO 'lost' during myocardial I/R injury.

### **1.6 Use of organic NO-releasing compounds on I/R injury**

Strategies that provide exogenous, synthesized sources of NO have been used for medical purposes for more than 120 years before the discovery of endogenous NO synthesis. As a result it was organic nitrates that were first used to attempt to replace deficiency of NO in I/R injury. The effects of different organic nitrate

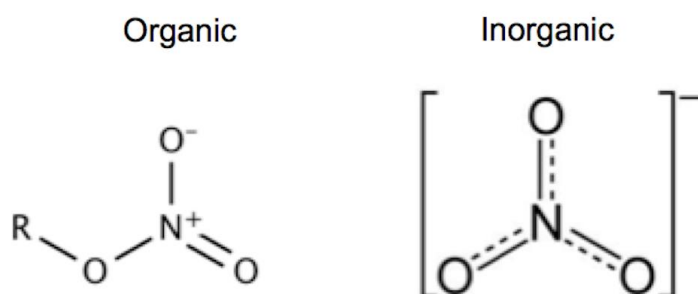
treatments in patients with AMI have been tested in 2 large scale trials of over 80,000 patients, receiving recommended therapies (90% aspirin, 70% fibrinolysis). Both trials showed that routine organic nitrate use did not produce an improvement in survival either in the total population or in subgroups (GISSI-3, 1994; ISIS-4, 1995). In the ISIS-4 study, 58,050 patients were randomized within 24 hours of AMI in a 2 x 2 x 2 factorial design to either isosorbide mononitrate (ISMN) (1 month of oral controlled-release mononitrate-30 mg initial dose titrated up to 60 mg once daily) versus matching placebo, oral captopril (6.25mg bd for 1 month) versus matching placebo or 24 hours of intravenous magnesium sulphate (8mmol). In those treated with ISMN, there was no significant reduction in 5-week mortality, either overall (2129 [7.34%] mononitrate-allocated deaths vs 2190 [7.54%] placebo) or in any subgroup examined (including those receiving short-term non-study intravenous or oral organic nitrates at entry). Further follow-up did not indicate any later survival advantage and there was an increase of 15 (SD 2) per 1000 events of hypotension (ISIS-4, 1995). The 2<sup>nd</sup> large study GISSI-3 trial, randomized 19,394 patients to a 24-hour infusion of glyceryl trinitrate (GTN) starting within 24 hours of pain onset, followed by topical GTN (10mg daily for 6 weeks) or to control. No difference in mortality was seen at 6 weeks in the group randomized to GTN therapy compared to control (6.52% vs 6.92%), with a similar result in the pre-specified combined endpoint of death plus left ventricular dysfunction or clinical heart failure (15.9% vs 16.7%) (GISSI-3, 1994). Further smaller trials assessing nicorandil and the NO donor molsidomine also failed to show any benefit (ESPRIM 1994; Kitakaze et al., 2007). The J-WIND study of 545 patients with AMI undergoing reperfusion therapy, randomized to receive either intravenous

nicorandil (0.067 mg/kg as a bolus, followed by 1.67 µg/kg per min as a 24-h continuous infusion) or placebo (0.9% saline). No reduction in infarct size (assessed by CK) and no improvement in either LVEF or outcome (mortality or MACE) was seen in the nicorandil group (Kitakaze et al., 2007). A further trial, ESPRIM of the NO donor molsidomine also failed to show any mortality benefit in AMI patients. In this trial, patients were randomly assigned to receive linsidomine 1 mg/hour intravenously for 48 h, followed by 16 mg molsidomine by mouth daily for 12 days (n = 2007), or an identical placebo (n = 2010). The molsidomine and placebo groups showed similar all-cause 35-day mortality (8.4% vs 8.8%) and long-term mortality (mean follow-up 13 months; 14.7% vs 14.2% p = 0.67) (ESPRIM Study group, 1994).

Although both organic and inorganic nitrates/nitrites mediate their principal effects via NO, there are many important differences. Inorganic nitrate and nitrite have simple ionic structures, are produced endogenously and are present in the diet, whereas their organic counterparts are far more complex, and, with the exception of ethyl nitrite, are all medicinally synthesised products (Figure 1.9). In addition the use of the organic nitrates are limited by the development of tolerance (Mayer and Beretta, 2008). Both of these effects are attributed to the tendency of the organic nitrates to increase the formation of damaging ROS. Rabbit aortae from animals treated with transdermal GTN for 3 days have double the amount of  $O_2^-$  (as measured by lucigenin chemiluminescence) as control animals (Munzel et al., 1995) and elevated levels of  $ONOO^-$  (assayed by nitrotyrosine accumulation) (Skatchkov et al., 1997). In marked contrast, pre-clinical evidence using inorganic  $NO_2^-$  as a source of NO have been associated



with a reduction in markers of oxidative stress (Tripatara et al., 2007) and studies in primates demonstrate no evidence of development of tolerance with repeated use (Dejam et al., 2007). Using inorganic nitrite to replace the NO lost during I/R injury could therefore be a better option.



**Figure 1.9. Chemical structure of organic and inorganic nitrate showing the difference between the molecules.**

### 1.7 Inorganic Nitrite ( $\text{NO}_2^-$ )

The biological role of the nitrite anion has undergone a significant transformation in recent years. For many decades, endogenous nitrite was considered simply a by-product of NO metabolism with limited functionally significant chemistry. However it is now recognized that it operates as a major storage form of NO in blood and tissues that can be reduced readily to NO to initiate cytoprotective signaling during pathological states including myocardial I/R injury (Zweier et al., 1995; Webb et al., 2004; Bryan NS, 2007). Systemic nitrite levels (100–500 nM in plasma (Dejam et al., 2005; Webb and Ahluwalia, 2010) and from 1-20  $\mu\text{M}$  in tissue (Bryan et al., 2005) are derived from both endogenous and exogenous sources with as much as 70% of plasma nitrite levels originating from the oxidation of eNOS-derived NO (Kleinbongard et al., 2003) and 30% proposed to

originate from dietary sources (Lundberg and Govoni, 2004; Lundberg et al., 2004) In general, the dietary component of plasma nitrite levels derives from inorganic nitrate. Nitrate enters the oral cavity where it is converted into nitrite by commensal bacteria in the mouth although evidence suggests this can also take place in the gastrointestinal tract (Zobell, 1932; Tannenbaum et al., 1976; Spiegelhalder et al., 1976; Lundberg and Govoni, 2004). Both sources of nitrite i.e. via the enterosalivary circuit and through oxidation of NO are important for maintaining steady-state nitrite levels in the plasma and tissues (Lundberg JO and Weitzberg E, 2008; Kapil et al., 2013).

The functional effects of nitrite in the cardiovascular system were first shown in the 1950s when Robert Furchgott demonstrated that high supraphysiological concentrations (high mM) of nitrite exerted relaxant effects on vascular smooth muscle (Furchgott and Bhadrakom, 1953). Despite this finding being over half a century ago, it was felt for many years that at physiological concentrations, nitrite was biologically inert and may even have deleterious effects with putatively carcinogenic properties (Tannenbaum and Correa, 1985). In 1995, evidence challenging this view began to emerge. Zweier and colleagues suggested that bioactive NO could be produced from physiological concentrations of nitrite under ischaemic and acidotic conditions in tissue of the cardiovascular system (Zweier et al., 1995). They performed studies using rat myocardium that clearly demonstrated NOS independent generation of NO detected with EPR spectroscopy under ischaemic and acidotic conditions. Following this definitive evidence of nitrite activity at physiological levels was provided in 2001, in rat aortic rings where low  $\mu\text{M}$  concentrations of nitrite, whilst inactive under

physiological pH, were shown to relax contracted rat aorta under acidic pH (6.6) conditions (Modin et al., 2001). Confirmation that nitrite affected vascular tone under physiological conditions was provided by studies by Gladwin and colleagues where the infusion of physiological levels of sodium nitrite into the human forearm caused vasorelaxation with concomitant increased blood flow (Cosby K, 2003). Importantly this phenomenon was augmented by the mildly hypoxic conditions associated with exercise confirming the notion that nitrite bioactivation is increased in the acidotic and hypoxic environment (Cosby K, 2003). More recently it has been suggested that endogenous  $\text{NO}_2^-$  reduction under physiological conditions does occur but that this is likely occurring to a greater extent on the venous side of the circulation where  $\text{O}_2$  tension is lower compared to the arterial side of the circulation (Maher et al., 2008).

Since publication of the above findings substantial research effort has focused on identifying the biochemical pathways involved in mammalian nitrite reduction. It is now clear that the conversion of nitrite to NO increases with increasing acidosis and hypoxia (van Faassen et al., 2009) environments in which the conventional L-arginine/eNOS pathway is dysfunctional (Giraldez et al., 1997; Abu-Soud et al., 2000). This particular finding has led to the proposal that this alternative pathway for NO generation may act as a complementary, back-up system when conventional NO generation has been compromised (Lundberg JO and Weitzberg E, 2008; Reutov, 2002). The exact mechanism and location for nitrite reduction in blood and tissues and its physiological role in regulation of cellular processes have not yet been pinpointed despite many possible targets, but it is clear from animal experiments that administration of nitrite (or its

precursor nitrate) has a therapeutic potential especially in I/R injury.

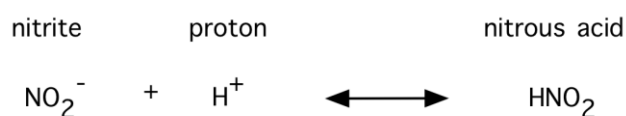
## **1.8 Conversion of nitrite to NO**

The reductive conversion of nitrite to NO is thought to occur by a number of mechanisms, including the enzymatic reductive actions of a variety of mammalian enzymes such as xanthine oxidoreductase (XOR) and non-enzymatic acidic disproportionation (Zweier et al., 1995). Importantly, environmental conditions play a major role in determining the extent of nitrite reduction with the bioconversion of nitrite to NO favoured under the hypoxic and acidic conditions present during ischaemia.

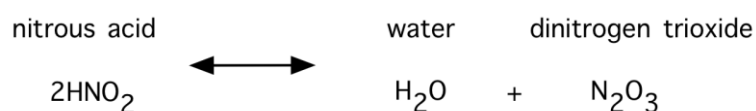
### **1.8.1 Non-enzymatic disproportionation**

NOS independent generation of NO was first described to occur in the stomach back in 1994 where two independent groups showed that NO and other reactive nitrogen oxides were generated non-enzymatically in large amounts following protonation in the stomach of nitrite in swallowed saliva (Lundberg et al., 1994; Benjamin et al., 1994). Soon after the discovery of gastric NOS-independent NO generation, Zweier and colleagues performed their landmark studies demonstrating profound NO generation in rat ischaemic heart muscle, which could not be effectively blocked by pharmacological NOS inhibitors (Zweier et al., 1995). During global cardiac ischaemia tissue pH fell below 6 and Zweier & colleagues demonstrated that under these conditions reduction of nitrite to NO was greatly enhanced.

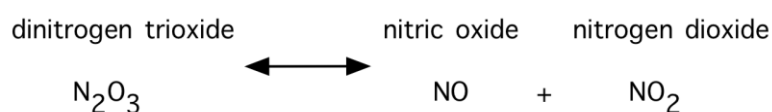
The chemical reduction of nitrite to NO follows several steps, including the intermediate nitrous acid HNO<sub>2</sub> (Equations 1.2a-c)



**Equation 1.2a NO<sub>2</sub><sup>-</sup> acidification to form HNO<sub>2</sub>**



**Equation 1.2b. Dehydration of HNO<sub>2</sub>**



**Equation 1.2c. N<sub>2</sub>O<sub>3</sub> dissociation to NO**

## 1.8.2 Enzymatic conversion

Haemoglobin (Cosby et al., 2003), myoglobin (Hendgen-Cotta et al., 2008), neuroglobin (Tiso et al., 2011), XOR (Webb et al., 2004), aldehyde oxidase (Li et al., 2008), eNOS (Gautier et al., 2006; Webb et al., 2008), and mitochondrial enzymes have all been identified with having a role in nitrite bioactivation. The relative contribution from these pathways varies between tissues based on the enzymatic composition within each tissue type. In the heart and specifically implicated in myocardial I/R injury there are two pathways that appear to reduce significant quantities of nitrite to NO, namely deoxymyoglobin (deoxyMb) (Shiva et al., 2007a; Rassaf et al., 2007) and XOR (Webb et al., 2004). This compares to

other compartments such as blood, where deoxyhaemoglobin (deoxyHb) has been implicated (Cosby et al., 2003). Interestingly in the heart, it has been shown that nitrite is still protective in a haemoglobin-free buffer isolated heart I/R model (Dezfulian et al., 2007) suggesting that haemoglobin likely plays a minor role in nitrite reduction in the myocardium despite its seemingly important role in the blood. The rest of this section will focus on the 2 key pathways that appear important in nitrite reduction in myocardial I/R injury, XOR and myoglobin.

#### **1.8.2.1 XOR as a myocardial nitrite reductase**

XOR is a molybdoflavin enzyme found in mammalian cells that is involved in the catabolism of purine and pyrimidines, oxidizing hypoxanthine to xanthine and xanthine to uric acid. Functional XOR is a homo-dimer of ~300 kD with each subunit consisting of four redox centers: a molybdenum cofactor (Mo), one FAD (Flavin adenine dinucleotide) site and two iron-sulfur ( $\text{Fe}_2/\text{S}_2$ ) clusters with purine oxidation being shown to occur at the Mo site. XOR also reduces oxygen to  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$  at the FAD site and is acknowledged as one of the key enzymes responsible for  $\text{O}_2^-$ -mediated cellular injury. As discussed previously in section 1.3.1, XOR is a key generator of ROS in myocardial I/R injury significantly contributing to cellular damage, an association confirmed by models where XOR inhibition leads to a reduction in symptoms and measurable restoration of function. This long-standing paradigm has been challenged with the demonstration of a nitrite reductase function for XOR suggesting XOR to be a source of beneficial NO under these same hypoxic/inflammatory conditions.

The reduction of nitrite to NO is catalyzed by XOR under hypoxic conditions, when electrons are supplied by either xanthine or NADH (Godber et al., 2000) (Li et al., 2001). This reduction occurs at the reduced Mo site and electrons driving this reaction can be supplied directly by xanthine or indirectly by NADH, via electron donation at FAD with subsequent retrograde flow to the Mo site (Li et al., 2001).

The role of XOR in nitrite reduction in the heart has been shown in several studies. Webb and colleagues showed that conversion of nitrite to NO was reduced by 50% with inhibitors of XOR, allopurinol (100 $\mu$ M) and BOF-4272 (10 $\mu$ M) in rat/human heart homogenates and almost completely abolished in the working heart (Webb et al., 2004). These findings were substantiated by further studies in rat heart homogenates where nitrite derived NO production was inhibited by 30% by the active metabolite of allopurinol, oxypurinol (100 $\mu$ M) (Liu et al., 2007) and in rat kidney homogenates where allopurinol (100 $\mu$ M) inhibited nitrite reduction by 71% (Tripatara et al., 2007).

#### **1.8.2.2 Myoglobin as a NO<sub>2</sub><sup>-</sup> reductase**

The monomer myoglobin is a small (17.6 kDa) but important intracellular oxygen binding haem protein. It is present at high  $\mu$ M concentrations in cardiac and skeletal muscle (Wittenberg and Wittenberg, 2003) and has been identified to have a role in nitrite bioactivation, specifically in myocardial I/R injury. Depending on ambient oxygen level, myoglobin is thought to act either as an NO scavenger under normoxic conditions or as a nitrite reductase under conditions

of hypoxia and ischaemia (Shiva et al., 2007a; Rassaf et al., 2007) Because myoglobin must be at least partially deoxygenated to act as nitrite reductase, this only occurs when the oxygen level falls below the P50 of myoglobin (Crawford et al., 2006).

Myoglobin has a high affinity for oxygen and a low haem redox potential that contributes to rapid nitrite reduction to NO when deoxygenated; in fact, deoxymyoglobin will reduce nitrite to NO at a rate 30-times faster than haemoglobin (Shiva et al., 2007a). These chemical properties suggest that when myoglobin becomes deoxygenated, such as in the subendocardium of the heart or in exercising skeletal muscle, it will rapidly convert nitrite to NO. This is done in much the same way as haemoglobin has been described to bioactivate nitrite (Equation 1.2) (Huang et al., 2005; Shiva et al., 2007a).



**Equation 1.3. Reduction of NO<sub>2</sub><sup>-</sup> by myoglobin or haemoglobin**

Studies in the heart have suggested that approximately 60%-70% of NO generation from nitrite may be mediated by the nitrite reductase activity of deoxymyoglobin, with the balance dependent on reduction by XOR (Shiva et al., 2007a; Rassaf et al., 2007). In agreement with an important role for myoglobin, nitrite reduction to NO is abolished in the myoglobin knockout mouse (Hengden-Cotta et al., 2008). Hengden-Cotta and colleagues subjected myoglobin knockout mice to a MI via coronary artery occlusion in the presence and absence of nitrite. While nitrite treatment decreased infarct size and improved post-ischaemic left



ventricular developed pressure in wild-type mice, nitrite did not mediate cytoprotection in myoglobin deficient mice. These studies suggest that nitrite and myoglobin play an important role in regulating cardiac energetics and oxygen utilization under conditions of physiological hypoxia. Whether myoglobin is also critical in nitrite reduction in the human heart is not known.

### **1.9 Pre-clinical evidence: Cytoprotective actions of nitrite in myocardial I/R injury**

Nitrite has now been shown to mediate a number of beneficial effects in the cardiovascular system. The most potent and reproducible of which is the ability of nitrite to mediate cytoprotection after I/R in a number of organs and species (Webb et al., 2004; Duranski MR et al., 2005; Pluta et al., 2005; Jung KH et al., 2006; Tripatara et al., 2007). The first demonstration of the cytoprotective effects of nitrite came in 2004 by Prof Ahluwalia's group (Webb et al., 2004) where administration of nitrite (10 or 100µm/l) either prior to or at reperfusion, in the isolated rat langendorff heart preparation improved both left ventricular function and coronary perfusion pressure as well as decreasing infarct size following an I/R insult. Several studies have followed confirming these beneficial effects in the heart using both in vitro (Baker JE et al., 2007; Hendgen-Cotta et al., 2008) but also in vivo pre-clinical rodent models of AMI (Duranski MR et al., 2005; Bryan NS, 2007). Duranski and colleagues in a model of AMI (left main coronary artery occlusion) administering the intra-ventricular injection of 2.4-1920nmol of nitrite 5 minutes prior to reperfusion, following 30 minutes of ischaemia, observed a dose-dependent decrease in infarct size with a maximal protective effect at 48

nmol, although the administration of 1920 nmol of nitrite failed to exert any significant cardioprotective effect (Duranski MR et al., 2005). Bryan and colleagues used a mouse model of AMI (left main coronary artery occlusion) and demonstrated that dietary nitrite supplementation (50mg/l) for 7 days prior to ischaemia resulted in a 48% reduction in infarct size (Bryan NS, 2007).

The functional benefits of nitrite have also been demonstrated in larger species. In a canine model of AMI induced by ligation of the LAD coronary artery in vivo, the intravenous administration of nitrite (0.2 $\mu$ mol/min/kg for 1<sup>st</sup> 20 minutes and then 0.17 $\mu$ mol/min/kg for 40 minutes) for the last hour of a 2 hour period of ischaemia, reduced infarct size by ~50% compared with control (Gonzalez et al., 2008). Importantly for clinical translation, the intravenous administration of nitrite during the last 5 minutes (0.2 $\mu$ mol/min/kg) of the 2-hour occlusion reduced infarct size and apoptosis almost as much as the 60-minute infusion. Such observations suggest that nitrite delivered just prior to reperfusion at the time of primary PCI could result in infarct size reduction.

Although the largest body of evidence demonstrating the cytoprotective properties of nitrite has accumulated in models of AMI, similar protective effects have been evidenced in other organs including the liver (Duranski MR et al., 2005), brain (Pluta et al., 2005) and kidney (Tripathara et al., 2007)(Table 1). For example, in a murine model of hepatic I/R, the intraperitoneal administration of sodium nitrite (1.2-480 nmol) during the ischaemic period exerted dose-dependent cytoprotective effects on hepatocellular necrosis and apoptosis as well as attenuating serum elevations of liver transaminases (Duranski MR et al.,

2005). In a rat model of cerebral I/R, 48 and 480 nmol of nitrite, infused intravenously at the start of reperfusion, exerted dose-dependent neuroprotective effects, reduced cerebral infarct volume by 33% and 77%, respectively, and promoted neurologic functional recovery (Jung KH et al., 2006). Similarly, the topical administration of sodium nitrite (0.12nmol/g) in a rat model of renal I/R injury, 1 minute before reperfusion significantly attenuated renal dysfunction, reperfusion injury, glomerular dysfunction and tubular injury (Tripatara et al., 2007). Interestingly in this study the intravenous administration of the same dose of sodium nitrite at the same time-point (1 minute before reperfusion) did not lead to an improvement in renal dysfunction or reperfusion injury (Tripatara et al., 2007). In all of these studies the beneficial effects were shown to be due to the activity of NO and were most often specifically associated with the local application into or on the organ of interest.

With the establishment of primary PCI as the gold standard treatment for AMI and evidence of the beneficial effect of the local administration of nitrite, this raises the question of the optimal route for the administration of nitrite therapy either intravenously like the canine AMI model (Gonzalez et al., 2008) or intra-coronary at the time of balloon inflation during primary PCI. When the two routes have been directly compared i.e in the kidney; the local administration of nitrite was effective at reducing infarct size whereas the same intravenous dose was not, suggesting a superiority of local administration (Tripatara et al., 2007). The intravenous route was effective in models of myocardial I/R injury including the most relevant to primary PCI (Gonzalez et al., 2008) however the intra-coronary route has several advantages, in that it would achieve a high local concentration

of nitrite within the desired area only; the myocardium, minimising the potential systemic side-effects that could occur (hypotension, methaemoglobinaemia). Additionally, if agents are to be delivered at reperfusion i.e. following angiography to ensure TIMI flow  $\leq 1$  the intra-coronary route will not delay reperfusion. This is compared to intra-venous administration, which may result in a delay in reperfusion whilst administered and also might require the establishment of very high circulating levels to achieve sufficient levels of nitrite. Together these observations suggest that nitrite may have use in the treatment of acute STEMI in which nitrite could be delivered locally (intra-coronary) before balloon inflation at the time of primary PCI.

**Table** Pre-clinical models of I/R injury  
**1.1**

<i>Organ</i>	<i>I/R model</i>			<i>Species</i>	<i>Placebo</i>	<i>Dose of nitrite, route, timing of administration</i>	<i>Timing of ischemia and reperfusion</i>	<i>Outcome measures of cytoprotection</i>	<i>Reference</i>
Heart	Isolated perfused heart (Langendorff)		heart	Rat	Saline	-10 or 100 $\mu\text{mol/L}$ -infusion -15 min before	30 min ischemia 120 min reperfusion	Infarct size Left ventricular pressure Rate of change of pressure during diastole	Webb et al. 2004
	Left main coronary occlusion		artery	Mouse	Nitrate saline or	-2.4-1920 nmol -intraventricular injection -5 min before reperfusion	30 min ischemia 24 reperfusion	Infarct size	Duranski et al. 2005
	Left main coronary occlusion		artery	Mouse	Saline	-48 nmol bolus -intraperitoneal injection -24 h or 5 min before reperfusion -200 $\mu\text{L}$ of 100 mg/L - oral gavage -24 h before ischemia onset	30 min ischemia 24 reperfusion	Infarct size	Shiva et al 2007
	In vivo-left anterior descending coronary artery occlusion			Rat	Nitrate saline or	In vivo- -0.04-10.0 mg/kg -bolus iv injection -15 min before ischemia onset	In vivo- 30 min ischemia	Infarct size	Baker et al 2007
	In vitro-isolated perfused heart (Langendorff)					In vitro- -1.0-300.0 $\mu\text{mol/L}$ -infusion -15 min before ischemia onset		Left ventricular developed pressure	
	Left main coronary occlusion Left anterior descending coronary artery occlusion		artery artery	Mouse Dog	Standard chow Saline	-Supplemented in drinking water (50 mg/L for 7 d) -0.20 $\mu\text{mol/min}$ per kg infusion for 20 min followed by 0.17 $\mu\text{mol/min}$ per kg infusion for 40 min or 0.20 $\mu\text{mol/min}$ per kg infusion for 5 min -during last 60 min or 5 min of ischemia, respectively	30 min ischemia 24 h reperfusion 2 h ischemia 6 h reperfusion	Infarct size Infarct size Microvascular perfusion Cardiomyocyte apoptosis Left ventricular ejection fraction	Bryan et al. 2007 Gonzalez et al. 2008
Liver	Hepatic occlusion	artery/portal	vein	Mouse	Nitrate saline or	-1.2-960 of nmol -intraperitoneal injection -midway through ischemia	45 min ischemia 5 (serum transaminase levels) or 24 h reperfusion	AST or ALT Hepatocellular necrosis and apoptosis	Duranski et al. 2005
	Hepatic occlusion	artery/ portal	vein	Mouse	Saline	-48 nmol of bolus -intraperitoneal injection -24 h before reperfusion or midway through ischemia -200 $\mu\text{L}$ of 100 mg/L oral gavage -24 h before ischemia onset	45 min ischemia 5 h reperfusion	AST or ALT	Shiva et al. 2007
Kidney	Bilateral renal pedicle occlusion			Rat	Saline	-0.12 nmol/g -administered topically or intravenously -1 min before reperfusion	60 min ischemia 6 h reperfusion	Serum creatinine, creatinine clearance, AST Renal injury (histology)	Tripatara et al. 2007 Milsom et al 2009
				Mouse	Saline				
Brain	Middle cerebral artery occlusion			Rat	Nitrate	-48-4800 nmol -intravenous infusion -1 min at start of reperfusion	90 min ischemia 3 h (LCBF) or 24 h (infarct size) or 2 h/1, 3, 7 d (neurologic testing) reperfusion	Infarct size LCBF Neurologic scores	Jung et al. 2006

### **1.10 Clinical evidence for inorganic nitrite**

Prior and during the tenure of this PhD, there had been no clinical trials of nitrite therapy for organ I/R injury conducted in man. However, the effects of nitrite have started to be translated into humans. Studies performed in healthy volunteers have shown that elevations in circulating nitrite levels (achieved through administration of a high dietary nitrate load) is associated with a number of beneficial effects including prevention of the development of transient endothelial dysfunction, in a model of I/R injury in the forearm (Webb et al., 2008) (Kapil et al., 2010). This is in addition to other studies demonstrating that an intra brachial infusion of nitrite vasodilates the human forearm without the development of tolerance and with increased activity in hypoxia (Cosby et al., 2003). Recently a study looking at the safety of sodium nitrite infusion in healthy subjects has demonstrated that it can be safely infused intravenously at defined concentrations for prolonged intervals (48 hours) (Pluta et al., 2011). Maximally tolerated doses for intravenous infusion were 267 mg/kg/hour with dose limiting toxicity occurring at 446 mg/kg/hour. Toxicity included a transient asymptomatic decrease of mean arterial blood pressure (more than 15 mmHg) and/or an asymptomatic increase of methaemoglobin level above 5% (Pluta et al., 2011).

Currently sodium nitrite is an FDA-approved drug being the active ingredient in cyanide antidote kits; in this indication the dose of nitrite administered to humans (500-1000 $\mu$ mol) is significantly higher than doses thought to be required to reduce I/R injury (0.5-10 $\mu$ mol). The acute toxicity issues lie with

respect to methaemoglobinemia (National Institute of Environmental Health Sciences, 1970), which has been associated with high doses of ingested nitrite, and it is this reaction/process (nitrite reacting with haemoglobin to form methaemoglobin and nitrate (section 1.5.2, equation 1.1) by which nitrite has been used to treat cyanide poisoning. However, results from data in humans demonstrate that vasodilating concentrations of nitrite (Cosby et al., 2003) do not induce clinically significant methaemoglobinaemia (Pluta et al., 2011).

### **1.11 Potential mechanisms for nitrite-induced cytoprotection in I/R injury**

While the exact mechanisms by which nitrite confers cyoprotection are not clear there are several potential theories that have been proposed. It is clear the beneficial effects of nitrite are dependent upon its conversion to NO, as evidenced by the complete block of nitrite induced protection by the NO scavenger (carboxy-PTIO) (Webb et al., 2004; Duranski MR et al., 2005). The mechanisms also appear to be independent of NO-synthases as cytoprotection induced by nitrite is not prevented by NO-synthase inhibition (Webb et al., 2004; Duranski MR et al., 2005; Lu et al., 2005) or affected in eNOS knockout models (Duranski MR et al., 2005). Different nitrite reductases appear to be responsible for NO formation during hypoxia, depending on the tissue involved and the severity of hypoxia. In the myocardium as discussed earlier it appears that the 2 key nitrite reductases are XOR and myoglobin. The involvement of XOR in the reduction of nitrite to NO appears important in the myocardium on the basis of reduced efficacy after treatment with allopurinol (Webb et al., 2004) whilst it has also been shown that the protective effect of nitrite was lost in myoglobin knockout

mice (Shiva et al., 2007a; Shiva et al., 2007b).

The derived NO or nitrite itself may modulate cellular proteins or lipids or may directly participate in actions which mitigate the injury occurring at reperfusion. Potential mechanisms for cytoprotection include the S-nitrosation of critical regulatory thiols on proteins such as caspase-3, a key constituent of the apoptotic pathway, this leads to its inactivation and prevents apoptotic cell death (Maejima et al., 2005), or the L-type calcium channel which results in reduced cytosolic  $\text{Ca}^{2+}$  and subsequent I/R injury (Sun et al., 2006).

#### **1.11.1 Improved mitochondrial function**

NO is a well-characterized regulator of mitochondrial function that stimulates mitochondrial biogenesis through the activation of sGC and the production of cGMP (Nisoli et al., 2005; Clementi and Nisoli, 2005). Therefore the mitochondrion has emerged as a major sub-cellular target of nitrite with accumulating evidence demonstrating that nitrite differentially regulates mitochondrial function through the modulation of specific proteins within the organelle in both physiology and pathology (for review see Murillo et al., 2011). The direct inhibition of complex 1 of the electron transport chain with resultant decrease in the amount of ROS formed, decreased  $\text{Ca}^{2+}$  influx and a reduction in cytochrome c release (Shiva et al., 2007b) has been implicated in nitrite mediated cytoprotection after I/R injury in both the heart and the liver (Gladwin et al., 2005). This inhibition of complex I may also prevent opening of the mPTP (Fontaine et al 1998; Nadtochiy et al., 2007) and appears to make mitochondria



more tolerant to  $\text{Ca}^{2+}$  overload (Nadtochiy et al., 2007). Nitrite via conversion to NO also results in the stimulation of sGC to form cGMP which in turn activates PKG resulting in stabilization of mitochondrial  $\text{K}_{\text{ATP}}$  channels (Sasaki et al., 2000). This stabilization is associated with decreased mitochondrial  $\text{Ca}^{2+}$  accumulation, and the prevention of mPTP opening (Takuma et al., 2001; Brown and Borutaite, 2002). The reversible inhibition of cytochrome c oxidase (complex IV of the electron transport chain) has also been implicated in nitrite mediated protection. NO is known to bind to this enzyme, in competition with oxygen, leading to the reversible inhibition of oxygen consumption (Poderoso et al., 1998). This inhibition of complex IV has consequences for the preservation of high-energy phosphate stores during I/R injury and may underlie the phenomenon of “myocardial hibernation” where metabolic activity of the heart is temporarily shut down during ischaemia in order to preserve high-energy phosphate reserves (Hendgen-Cotta et al., 2008; Murillo et al., 2011).

As stated above it is clear that the beneficial effects of nitrite are dependent upon its conversion to NO, however recently in vivo and in vitro data has emerged to suggest that nitrite itself may also have independent effects on mitochondrial function (Mo et al., 2012; Kamga Pride et al., 2014). Mo and colleagues demonstrated in a rat model of carotid injury, that two weeks of continuous oral nitrite treatment post-injury prevented the hyperproliferative response of smooth muscle cells, accompanied by a nitrite-dependent upregulation of PGC1 (the master regulator of mitochondrial biogenesis) and increased mitochondrial number in the injured artery. In vitro they demonstrated that this increase in mitochondrial number did not require activation of sGC and was due to nitrite

augmenting the activity of adenylate kinase, resulting in AMP kinase phosphorylation, downstream activation of sirtuin-1, and de-acetylation of PGC1 (Mo et al., 2012). Additionally and more specific to cardioprotection, Kamga-Pride and colleagues using a model of hypoxia/reoxygenation in cultured rat H9c2 cardiomyocytes, demonstrated that a transient (30 minutes) normoxic nitrite treatment significantly attenuated cell death after a hypoxic episode initiated 1 hour later. As this was a normoxic treatment it is unlikely nitrite was converted to NO and mechanistically, it was demonstrated that this protection depended on the activation of PKA, which phosphorylated and inhibited dynamin-related protein 1, leading to the enhancement of mitochondrial fusion in normoxia. Functionally, this augmented mitochondrial  $O_2^-$  production, which oxidized and activated AMP kinase, an essential step in nitrite-mediated preconditioning (Kamga Pride et al., 2014).

By these proposed mechanisms nitrite has beneficial inhibitory effects on several of the key process implicated in reperfusion injury; ROS generation,  $Ca^{2+}$  overload and mPTP opening. Additionally nitrite also exerts a range of anti-inflammatory and anti-platelet effects common to NO, two key processes known to play important roles in myocardial I/R injury. As such these effects are discussed in more detail in the following sections.

### **1.12 Nitrite/NO and platelet reactivity**

Platelet adhesion and aggregation are important in the pathogenesis of AMI. The role of platelets, in terms of activation and aggregation, is well recognized as a terminal event in the formation of thromboses which cause blockages of arteries and lead to stroke, AMI and other vascular events (Davi and Patrono, 2007). Therefore, inhibiting platelet reactivity is an important strategy in the prevention and treatment of AMI. It is widely accepted that NO suppresses the adhesion of platelets to intact vascular endothelium (Radomski et al., 1987; Dangel et al., 2010) and also platelet aggregation itself (Radomski et al, 1990). The effect of NO has been attributed, primarily, to activation of sGC and consequent elevation of platelet cGMP levels (Moro et al., 1996; Dangel et al., 2010; Radomski et al., 1990) although, evidence also exists to suggest there may be sGC-independent effects of NO and NO donor drugs on platelets (Pawloski et al., 1998; Zhang et al., 2011).

The exact mechanisms in which cGMP elevation inhibits platelet aggregation are uncertain however it has been hypothesised that the elevation of cGMP in platelets results in PKG activation and consequent phosphorylation of a range of proteins that influence platelet function including inositol triphosphate receptors and PDE-5 (Jones et al., 2012). The importance of PKG is exemplified by studies with PKG-deficient mice demonstrating increased platelet adhesion and aggregation in vivo and lack of response to NO in vitro (Massberg et al., 1999). Irrespective of the exact molecular pathways involved, NO has been shown to influence all major aspects of platelet function including  $\text{Ca}^{2+}$  mobilization, shape change, secretion and both integrin activation and outside-in signalling to ensure

a coordinated modulation of platelet activity (Naseem and Roberts, 2011). Specifically NO is known to regulate P-selectin expression on activated platelets, an adhesion molecule important for inter-platelet aggregation (stabilizing the initial GPIIb/IIIa–fibrinogen interactions) and known to aid leukocyte and/or endothelial adhesion (Murohara et al., 1995; Michelson et al., 1996; Gries et al., 1998). The ability of NO to control such a diverse range of events is explained by the capacity of cGMP/PKG signaling pathways to interact with a plethora of target proteins.

A growing weight of evidence suggests that nitrite via its conversion to NO reduces platelet aggregation (Johnson et al., 1990; Richardson et al., 2002; Webb et al., 2008; Velmurugan et al., 2013). Ex-vivo incubation of platelet rich plasma with supraphysiological nitrite concentrations (60µM) reduced the aggregatory response to adenosine diphosphate (ADP), arachidonic acid and collagen whilst similar concentrations of inorganic nitrate had no effects (Schafer et al., 1980), as measured by light transmission aggregometry (Srihirun et al., 2012). In humans, oral supplementation with either 2mmol inorganic potassium nitrate (KNO<sub>3</sub>) (Richardson et al., 2002) or 500ml beetroot juice (providing ~22.5mmol nitrate) (Webb et al., 2008) reduced platelet aggregation to ADP and collagen. The importance of the bioactivation of nitrate to nitrite was confirmed with both the antiplatelet effect and rise in serum nitrite levels abolished by interruption of the entero-salivary circuit (Webb et al., 2008). In another study, acidified sodium nitrite has been demonstrated to significantly inhibit platelet aggregation in a concentration dependent manner in cat platelet-rich plasma (Johnson et al., 1990). Recent in-vitro experiments with human platelet rich plasma have also

demonstrated decreased platelet reactivity (as measured by P-selectin and GPIIb/IIIa expression) in response to sodium nitrite (0.1-1.0  $\mu$ M) treatment at oxygen levels lower than 49 mmHg. The inhibitory effect of nitrite was augmented by decreasing oxygen saturation and the platelet inhibition prevented by the addition of an NO scavenger (carboxy-PTIO) (Akrawinthawong et al., 2014). The precise mechanism(s) of this inhibition has still to be clarified. Nevertheless, the above studies have shown that nitrite has inhibitory effects on platelet activation, which could contribute to the potential beneficial effects of nitrite in AMI.

### **1.11 Nitrite/NO and inflammation**

NO plays an important role in modulating many aspects of the inflammatory response. In particular NO inhibits leukocyte recruitment (Kubes et al., 1991) (Ahluwalia et al., 2004), which is a key element in reducing the inflammatory cell-driven injury to the endothelium and perivascular myocardium (dPalazzo et al., 1998) associated with I/R injury. Leukocyte recruitment is a dynamic cellular and molecular process and constitutes leukocyte tethering, rolling, adhesion and ultimately emigration from the microvasculature (Petri et al., 2008). Previous work has demonstrated that NO is key in the regulation of interactions of the leukocyte with endothelial cells within the circulation (Kubes et al., 1991). Inhibition of endogenous NO production in postcapillary venules by L-NMMA or L-NAME increased leukocyte adhesion to the endothelium by 15-fold and reduced wall shear rate by half shown in a cat mesentery study. Additionally leukocyte adhesion was completely reversed by the addition of L-arginine and by

an antibody specific for the leukocyte adhesion molecule glycoprotein CD18 (Kubes et al., 1991).

NO appears to play an important role in regulation of P-selectin expression. NO reduces P-selectin expression, while inhibitors of NOS elicit an increase in P-selectin expression, and a corresponding increase in leukocyte adherence to the endothelium (Kubes et al. 1991; Armstead et al. 1997). As in the case of relaxant effects of NO on vascular smooth muscle, down-regulation of P-selectin expression by NO is mediated via sGC/cGMP (Ahluwalia et al. 2004).

To date there has been limited research investigating whether nitrite might influence inflammatory cell recruitment. In mice fed a high cholesterol diet, signs of inflammation were shown to be evident in intra-vital microscopy studies assessing leukocyte adhesion and emigration through the cremaster venular endothelium (Stokes et al., 2009). In these studies, nitrite supplementation (33 and 99mg/l in drinking water) inhibited leukocyte adhesion and emigration as well as reversing endothelial dysfunction. These results assessed in ex vivo organ bath experiments suggest a beneficial effect of nitrite administration in hypercholesterolaemia-induced inflammation. Jadert et al. studied the effects of both dietary nitrate and nitrite supplementation on acute microvascular inflammation (Jadert et al., 2012). In 2 separate experiments, they demonstrated that a dietary nitrate load (0.85 g/L or 2.0 mmol/kg/day for 7 days, given in the drinking water of mice) or intravenous sodium nitrite (1.3 mg/kg) decreased leukocyte recruitment after the induction of a predominantly neutrophil-mediated inflammatory response induced by the intra-peritoneal administration

of the neutrophil chemokine CXCL-2. Both the intravenous nitrite treatment and dietary nitrate intake affected various stages of leukocyte recruitment. Intravenous nitrite had significant effects on adherence and emigration but no effect on rolling whereas the dietary nitrate treatment affected emigration and rolling but not adherence. Both treatments were partly cGMP-dependent indicating a role for NO (Jadert et al., 2012). The study suggested that intravenous nitrite therapy (or oral nitrate) could potentially be a way to mitigate the detrimental effects of an acute inflammatory process.

There is sparse data assessing the effect of NO therapy on the inflammatory response in humans. A recent study using a human model of I/R injury showed an attenuation of the inflammatory response secondary to inhaled NO therapy (Mathru et al., 2007). In this study patients undergoing knee surgery were recruited and a tourniquet was applied during surgery to the upper portion of the leg for 2 hours to create a period of ischaemia. Inhaled NO (80 ppm) or placebo was administered before tourniquet application and continued throughout reperfusion until the completion of surgery. Reperfusion resulted in an inflammatory response locally in the skeletal muscle characterized by NF- $\kappa$ B activation and increased oxidative stress accompanied by enhanced systemic expression of adhesion molecules CD11b/CD18 and soluble P-selectin in peripheral blood. These alterations were significantly attenuated in patients who received inhaled NO (Mathru et al., 2007), suggesting a potential benefit in replacing NO in I/R injury.

### **1.12 Outcomes after PCI**

Patients undergoing the treatment of AMI with opening of the culprit (occluded) coronary artery have endovascular stents placed during the angioplasty procedure to prevent the vessel from collapsing or closing up again. A major limitation of this technique is re-growth of the tissue within the stented part of the artery, known as in-stent restenosis (ISR). Developments in the field of endovascular stents have substantially reduced the rates of ISR from 30% (with the use of bare metal stents) to 10% (with drug eluting stents (DES)) (Jukema et al., 2012b). However, in addition to these residual rates of ISR, concern has arisen about the potential for late or very late stent thrombosis after DES implantation, with extended-duration dual anti-platelet therapy now recommended in these patients {(Hamm et al., 2011; Nakazawa et al., 2008). After injury caused by balloon angioplasty or stent deployment in PCI the endothelium is partially or completely denuded (Harnek et al., 1999; Otsuka et al., 2012), triggering inflammatory mechanisms like platelet aggregation and formation, vascular smooth muscle cell (VSMC) migration and proliferation, extra cellular matrix (ECM) formation, and finally ISR (Jukema et al., 2012b). The significance of delayed re-endothelialization, inhibition of vascular repair and late stent thrombosis is particularly important following implantation of DES because the anti-proliferative agents used to prevent VSMC proliferation also delay re-endothelialization in the stented segment (Finn et al., 2007; Nakazawa et al., 2008). Importantly it has been shown that a deficiency in NO contributes to the delay in re-endothelialization following PCI and therefore the replacement of this could be beneficial. Recent evidence suggests that the ISR rate (assessed by late



loss and target vessel revascularisation) can be reduced by additional pharmacotherapy such as NO that is anti-inflammatory and anti-platelet in activity (Lee et al., 2008). If NO, which is known to have anti-inflammatory and anti-platelet effects (as discussed above) also has effects on endothelial cell growth promoting re-endothelization and VSMC growth inhibiting proliferation this could have important effects on long-term outcomes after PCI potentially reducing ISR and stent thrombosis.

### **1.13 NO, nitrite and cell growth**

NO is believed to play different roles in regulating the growth of different cell types involved in arterial injury. While it is believed to inhibit smooth muscle cell proliferation (Zuckerbraun et al., 2007; Zuckerbraun et al., 2010) it is thought to stimulate endothelial cell proliferation (Ziche et al., 1997; Cooke and Losordo, 2002; Metaxa et al., 2008). Thus nitrite derived NO might also be useful in modulating these cellular responses to injury.

#### **1.13.1 Endothelial cells**

NO is thought to stimulate endothelial cell proliferation and the regeneration of healthy endothelium (Ziche et al., 1997; Cooke and Losordo, 2002; Metaxa et al., 2008), however not all studies have shown a stimulating effect. There are studies suggesting NO inhibits endothelial cell growth (Heller et al., 1999), however these effects have been shown at very high (>100  $\mu$ M) concentrations of an NO donor with the consensus that at lower physiological levels NO not only enhances

endothelial cell proliferation but also promotes migration, angiogenesis (Cooke and Losordo, 2002), and inhibits endothelial cell apoptosis (Rossig et al., 1999). Convincing evidence of the effect of NO on cell growth is provided by the impairment of cell growth resulting from its inhibition. In lung epithelial and pleural mesothelial cells, the depletion of endogenous NO by the NO scavenger carboxy-PTIO stops cell proliferation by arresting the cell cycle at the late S or G2/M phases, events that are mediated by a cGMP-dependent pathway (Janssen et al 1998). Recently an in-vivo study showed that eNOS deficiency causes collateral vessel rarefaction, impairing the activation of a cell cycle gene network during arteriogenesis; suggesting a role for eNOS in maintaining native collateral density during growth to adulthood and in collateral remodeling in obstructive disease through regulation of cell proliferation (Dai and Faber, 2010).

Various mechanisms have been implicated in NO induced endothelial cell proliferation. In coronary venular endothelial cells, exogenous and endogenous NO both induce a proliferative response by a mechanism implicating basic fibroblast growth factor (bFGF) as an anti-bFGF antibody blocks NO-mediated proliferation (Ziche et al., 1997). In addition, the proliferative action of exogenous NO, via activation of the mitogen-activated protein kinase (MAPK) pathway, was also demonstrated in foeto-placental artery endothelial cells (Zheng et al 2006) suggesting a positive role of NO in angiogenesis. VEGF-mediated proliferation of umbilical vein endothelial cells and activation of Raf-1, the first kinase of the MAPK cascade are NO dependent processes controlled by cGMP and its target PKG (Hood and Granger 1998). NO enhances endothelial migration (Ziche et al., 1994; Murohara et al., 1999) by stimulating endothelial cell podokinesis (Noiri et

al., 1998), enhancing the expression of  $\alpha_v\beta_3$  (Murohara et al., 1999), and increasing dissolution of the extracellular matrix via the bFGF-induced upregulation of urokinase-type plasminogen activator (Ziche et al., 1997).

High levels of NO as well as inhibiting endothelial cell growth have also been shown to induce apoptosis however lower levels of NO function as an important inhibitor of apoptosis by interfering with signal transduction pathways that control apoptotic cell death (Dimmeler and Zeiher, 1997). Using cultured human umbilical vein endothelial cells (HUVECs) that had been subjected to shear stress in the presence of NO, Dimmeler et al demonstrated that NO (sodium nitroprusside [SNP] 10  $\mu$ M) prevented apoptosis (Dimmeler et al., 1997), and similar effects have also been demonstrated in porcine endothelial cell models (DeMeester et al., 1998). Tzeng et al showed that an adenoviral vector overexpressing iNOS caused an inhibition of lipopolysaccharide induced apoptosis in cultured sheep arterial endothelial cells by reducing caspase-3-like protease activity (Tzeng et al., 1997). Further clarification regarding this mechanism was provided by studies in human endothelial cells where NO supplied by exogenous NO donors (SNP or PAPA NONO-ate [50  $\mu$ M]) served as an anti-apoptotic regulator of caspase activity via S-nitrosation of the Cys-163 residue of caspase-3 (Rossig et al., 1999). Taken together, these results suggest that NO production favors healing of injured vasculature by inhibiting further apoptosis of endothelial cells in order to rapidly cover the injured site and therefore the provision of NO to these areas could be beneficial.

Data suggests that the provision of NO in the form of nitrite has beneficial effects

on endothelial cell proliferation and protection under hypoxia. Nitrite has been shown to protect endothelial barrier function against hypoxic challenge by regulating caspase-3 activity. Studies in zebrafish and murine endothelial cells showed that nitrite promotes S-nitrosation and consequent inactivation of caspase-3, leading to a sustained barrier function through better maintenance of cadherin junctions (Lai et al., 2011). Other studies have demonstrated that nitrite-mediated cell proliferation is via the induction of the S phase of the cell cycle (Kumar et al., 2011). Using human neutrophilic HL-60 cells it was also shown that nitrite induced augmentation in the S phase. In the same study, up-regulation of cell cycle regulators, efficient Cdk2/cyclin E interaction, and augmented Cdk2 activity were observed in nitrite-treated cells with the proliferative effect of nitrite blocked by roscovitine, a Cdk2 inhibitor (Kumar et al., 2011). Overall this suggested that the proliferative effect of nitrite on HL-60 cells seemed to be NO mediated, redox sensitive and Cdk2 activation dependent.

Despite the majority of evidence existing to suggest that nitrite exerts effects on endothelial cells under hypoxic conditions there has been at least one study demonstrating the effects of nitrite on cell growth under normoxic conditions. A recent study by Wang and colleagues (Wang et al., 2012) showed that sodium nitrite (1-100 $\mu$ M) enhances cell proliferation and subsequent wound healing of human airway epithelial cells in normoxic physiological conditions. Interestingly they also found that intracellular signaling after stimulation with nitrite under normoxia was independent of NO and cGMP generation, and involved oxidative reactions with hydrogen peroxide and downstream ERK1/2 activation. This suggests that nitrite may have a direct effect on endothelial cells rather than by

conversion to NO. Further data is required to assess whether nitrite has an effect on cellular proliferation in normoxia.

### **1.13.2 Smooth muscle cells**

NO has been shown to conversely limit smooth muscle cell proliferation and migration via both cGMP-dependent and independent mechanisms (Zuckerbraun et al., 2007) (Zuckerbraun et al., 2010). Early studies demonstrated that NO donor agents inhibit the proliferation of isolated rat and rabbit VSMCs in tissue culture (Kariya et al., 1989; Garg and Hassid, 1989; Assender et al., 1992; Mooradian et al., 1995). In several studies, the effects of low, but not high concentrations of NO-donors were mimicked by 8-Br-cGMP (Assender et al., 1992) indicating the involvement of cGMP-dependent protein kinases. However, NO can also directly inhibit the synthesis of RNA and proteins in VSMCs (Jeremy et al., 1999) and directly inhibit mitochondrial respiration, which, in turn, may influence cell growth (Giuffrè et al., 1996). Sarkar and colleagues (Sarkar et al., 1997b) suggested multiple sites for the effect of the NO donors, SNP and S-nitroso-glutathione (SNOG) on the cell cycle in rat aortic VSMCs. Individually, these elicited a 50% reduction in the fraction of cells in the S and G<sub>2</sub> 1M phases and a corresponding increase in the G<sub>1</sub> fraction, suggesting that NO inhibits S-phase entry of VSMCs. Addition of both donors together, however, immediately blocked replication reversibly in the S-phase, an effect that was not mimicked by exogenous cGMP. These experiments intimate that NO inhibition of VSMC proliferation is associated with two distinct and reversible cell cycle arrests, an immediate cGMP-independent S- phase block and a cGMP-dependent shift back

from the  $G_1$ -S boundary to a quiescent  $G_0$ -like state (Sarkar et al., 1997a; Sarkar et al., 1997b).

Subsequently, Ishida and colleagues used SNP-treated VSMC to demonstrate that NO halted the G1/S transition by a p21-mediated inhibition of the phosphorylation of the retinoblastoma protein by cyclin-dependent kinase 2 (Ishida et al., 1997). Further evidence for a role of p21, has been provided by studies using the gene transfer of iNOS into VSMC that demonstrated p53-independent and cGMP-independent increases in expression of p21 (Kibbe et al., 2000). Further verification has been provided by studies (Tanner et al., 2000) demonstrating that NO alters the expression or activity of many cell cycle regulatory proteins, including cyclin A, cyclin-dependent kinase 2, and p21. NO also retards VSMC migration, with the NO donor SNP shown to inhibit angiotensin II-induced rat VSMC migration in vitro, as measured by a modified Boyden Chamber (Dubey et al., 1995). In addition other NO donors, including diethylamine NONO-ate (DETA/NO), spermine NONO-ate (SPER/NO), and S-nitrosoglutathione, have all been shown to exhibit concentration-dependent inhibition of both the number of and distance migrated by VSMC, with the inhibition reversible upon removal of the NO donors (Sarkar et al., 1996).

It has been demonstrated that low dose sodium nitrite given prior to balloon injury in vivo prevents vascular intimal hyperplasia in both rat and mouse injury models (Alef et al., 2011). Moreover, it has been shown that nitrite delivered after the establishment of intimal hyperplasia could reverse the pathological process (Alef et al., 2011). In these studies sodium nitrite, was shown to be metabolized to

biologically active NO by XOR and to limit VSMC proliferation via a p21<sup>Waf1/Cip1</sup> dependent-mechanism. However not all studies have found an inhibitory effect of nitrite on VSMC growth. A study by Vavra and colleagues. demonstrated that neither nitrite nor nitrate inhibited rat aortic VSMC proliferation in vitro (Vavra et al., 2011). However both these studies did suggest a benefit of sodium nitrite in the treatment of/protection from restenosis in vivo (Alef et al 2011; Vavra et al., 2011)

Whether the effects of nitrite on cell growth might be of use in improving outcomes after stent placement in humans is unknown. If it can be demonstrated that nitrite protects endothelial cells from the effects of ischaemia and I/R injury promoting re-endotheliasation after PCI and also exert an opposing repressive influence over smooth muscle cell growth, it would suggest that in an environment where improved endothelial cell growth but repressed smooth muscle cell growth is desired, such as in restenosis following stent implantation, raising circulating nitrite levels may be of therapeutic utility.

### **1.14. Aims and Hypotheses**

The primary aim of my PhD is to test the hypothesis that inorganic nitrite protects patients undergoing primary PCI for the treatment of AMI against the effects of I/R injury.

Specific aims are:

- (i). To determine whether intra-coronary nitrite is safe, tolerable and reduces infarct size during primary PCI in patients with AMI.
- (ii). To understand the mechanisms by which nitrite reduces reperfusion injury, focusing specifically on inflammation and platelet activity.
- (iii). To investigate whether prolonged nitrite treatment might prove useful in promoting endothelial and vascular smooth muscle cell growth under normoxia, hypoxia and I/R injury and may therefore affect restenosis after PCI.



# **Chapter 2.0**

## **General Methods**

## **2.1 Clinical Trial**

The study performed employed a double-blind, randomized, single-centre, placebo-controlled trial design to determine whether intra-coronary nitrite injection reduces infarct size in patients with myocardial infarction undergoing primary angioplasty (NITRITE-AMI). Subjects were randomized to receive either an intracoronary bolus (10 ml) of sodium nitrite (1.8  $\mu$ mol in normal saline) or normal saline distal to the occlusion site before initial balloon inflation during primary PCI. The dose was chosen to achieve a local tissue concentration of 2.5-10  $\mu$ mol/L following bolus administration (Cosby et al 2003); a concentration associated with cardioprotection in pre-clinical studies (Webb et al 2004; Duranski et al 2005; Gonzalez et al 2008).

### **2.1.1 Study Protocol**

The study was performed with ethical approval from the National Research Ethics Service London – West London (Reference: 11/LO/1500) for ethical human research and sponsored by Barts Health NHS Trust Research and Development Department. MHRA approval was also obtained (EudraCT No:2011-000721-77). The trial was performed in accordance with the Declaration of Helsinki (revised version, 1996) and the principles of the International Conference on Harmonization–Good Clinical Practice (ICH-GCP) guidelines. All subjects gave written informed consent before being included in the study. Any serious adverse event or reaction during the course of the study was reported to the Barts Health NHS Trust Joint Research Office as per adverse event guidelines.

The study was registered with the United Kingdom Clinical Research Network (Study ID 12117) and ClinicalTrials.gov (NCT01584453)

### **2.1.2 Patient selection**

All consecutive patients presenting to Barts Health Heart Attack Centre, based at The London Chest Hospital, suspected of an acute STEMI and candidates for primary PCI were considered eligible for participation. Potentially eligible patients were approached at the time of their acute admission with STEMI. Due to limited time available a rapid assessment of preliminary eligibility was made and consent taken at the time of consent for the primary angioplasty procedure. Patients were given approximately 5-10 minutes to consider their participation and further opportunity was given to the patient to reconsider any decisions made at this time during the procedure. As a consequence of the short consideration time, patients with limited English were excluded. Members of staff obtaining this consent were GCP trained and aware of the Mental Capacity Act 2005 being able to assess the patient's capability to give consent in an emergency situation. The initial information provided to the patient included a summary patient information sheet (PIS) (see appendix 8.1), which provided adequate explanation of the aims, methods, anticipated benefits and potential hazards of the study. A detailed PIS (see appendix 8.2) was provided following the procedure. Entry into the trial was not confirmed until diagnostic angiography and the patient had fulfilled the pre-specified inclusion criteria (section 2.1.3) and did not meet any exclusion criteria (section 2.1.4).

### **2.1.3 Inclusion criteria**

Inclusion criteria were:

1. Patients aged between 18 and 80 years
2. Acute STEMI with ECG showing ST-segment elevation of 1mm or more in two adjacent limb leads or 2mm or more in at least two contiguous precordial leads or new left bundle branch block
3. Haemodynamically stable
4. Estimated symptom to balloon or thrombus aspiration time < 6 hours
5. Signed and dated written informed consent prior to study admission
6. Angiographically
  - i. Primary PCI indicated for revascularisation
  - ii. Single epicardial artery to be treated
  - iii. Expected ability to use over the wire balloon

### **2.1.4 Exclusion criteria**

Exclusion criteria were:

1. Inability to consent (including inability to speak English)
2. Patients on organic nitrate treatment (Nicorandil, ISMN)
3. Previous history of AMI, systolic dysfunction or CABG
4. Subjects presenting with cardiogenic shock (systolic blood pressure <80 mmHg for >30 minutes, or requiring inotropes or emergency intra aortic balloon pump for hypotension treatment) or cardiopulmonary resuscitation

5. Current diagnosis of or treatment for malignancy, other than non-melanoma skin cancer.
6. Current life-threatening condition other than vascular disease that may prevent a subject completing the study.
7. Use of an investigational device or investigational drug within 30 days or 5 half-lives (whichever is the longer) preceding the first dose of study medication.
8. Patients considered unsuitable to participate by the research team (e.g., due to medical reasons, laboratory abnormalities, or subject's unwillingness to comply with all study-related procedures).
9. Severe acute infection, or significant trauma (burns, fractures).
10. Pregnancy.
11. Contra-indications to CMR scanning
  - i. Pacemakers, intracranial clips or other metal implants
  - ii. Claustrophobia
  - iii. Renal failure (eGFR<30mls/min)
12. History of alcohol or drug abuse within the past 6 months
13. History of congenital methaemoglobinaemia
14. Angiographically, severe vessel tortuosity, diffuse disease or severe calcification is present which may impede successful delivery of the over the wire balloon

### **2.1.5 Randomisation**

After coronary angiography, patients were randomized (1:1) to a bolus injection of intracoronary sodium nitrite (1.8  $\mu$ mol in 10 mL of 0.9% NaCl) or placebo (10 mL of 0.9% NaCl). Manufacture of the nitrite and placebo, blinding, coding and randomisation were conducted by the pharmacy manufacturing unit at Ipswich Hospital prior to transfer of stock to the London Chest Hospital Pharmacy. The randomisation list was computer-generated based on blocks of ten to assign patients to treatment or placebo group and kept in a sealed envelope in the hospital pharmacy. A total of 80 indistinguishable vials of sodium nitrite and placebo were provided. All study personnel were blind to treatment allocation until the study had been completed and all analyses performed.

### **2.1.6 Investigational medicinal product (IMP)**

The experimental intervention was a bolus of sodium nitrite solution (1.8 $\mu$ mol in 10 ml (pre-diluted in 0.9% sodium chloride in a syringe) which was delivered over 30-60 seconds via intracoronary injection initiated during the re-establishment of antegrade epicardial flow with primary PCI. The control intervention was a bolus of 0.9% sodium chloride solution (prepared with an identical appearance to the sodium nitrite). This dose of nitrite (1.8  $\mu$ mol) was chosen since studies in the forearm of healthy volunteers demonstrates that this dose results in a local concentration of 2.5-10 $\mu$ mol/l (Cosby et al., 2003; Dejam et al., 2007; Maher et al., 2008). This dose sits within the range associated with cardioprotection in reperfusion injury achieved through bolus dose

administration in pre-clinical studies (Gonzalez et al., 2008; Duranski MR et al., 2005) and provides levels of nitrite that are approximately 10-100 times higher than circulating physiological levels which are thought to be between 0.02-0.4  $\mu\text{mol}$  (Webb and Ahluwalia, 2010)

The IMP was stored in the pharmacy at the London Chest Hospital with 6 vials stored in the controlled drug (CD) cupboard in catheterization laboratory one at the London Chest Hospital. This was done so that the IMP was readily deliverable to patients on their arrival to the hospital in the emergency setting. When the stock level fell to below 3 vials in the cupboard, a further 3 vials were obtained from the pharmacy. The CD cupboard was temperature monitored so that the IMP was kept between 15-25°C. No deviations from this range were noted during the trial period.

Following patient consenting and fulfilling of inclusion and no exclusion criteria the patient was randomized to receive the next sequential vial of IMP (i.e. Patient 010 received vial 010). The sodium nitrite was stored in volumes of 10ml at a concentration of 0.435M equating to 4350 $\mu\text{mol}$  in normal saline. Therefore it required serial dilutions to generate the correct delivery dose (1.8 $\mu\text{mol}$ ). Initially 2.1ml (913.5 $\mu\text{mol}$ ) was removed from the vial and made up to 50ml with normal saline, resulting in a concentration of 18.27 $\mu\text{mol}/\text{ml}$  (syringe A). Of this solution 0.5ml (9.135 $\mu\text{mol}$ ) was then removed and made up to 50ml with normal saline to give a concentration of 0.18 $\mu\text{mol}/\text{ml}$  (syringe B). Following this 10ml of this solution was then taken into a sterile 10ml syringe (C) (1.8 $\mu\text{mol}$  in 10ml) ready for delivery to the patient. The same dilution steps were performed for both

nitrite and placebo as the vials were indistinguishable from each other and the study personnel were blinded to treatment allocation.

<p>FOR CLINICAL TRIAL USE ONLY</p> <p>EudraCT reference: 2011-000721-77</p> <p>Placebo/Active Nitrite Dilution</p> <p>Subject Number:</p> <p>Date:</p> <p><b>Syringe: A</b></p> <p><b>Syringe A for dilution use only</b></p>	<p>FOR CLINICAL TRIAL USE ONLY</p> <p>EudraCT reference: 2011-000721-77</p> <p>Placebo/Active Nitrite Dilution</p> <p>Subject Number:</p> <p>Date:</p> <p><b>Syringe: B</b></p> <p><b>Syringe B contains final Injectable product</b></p>
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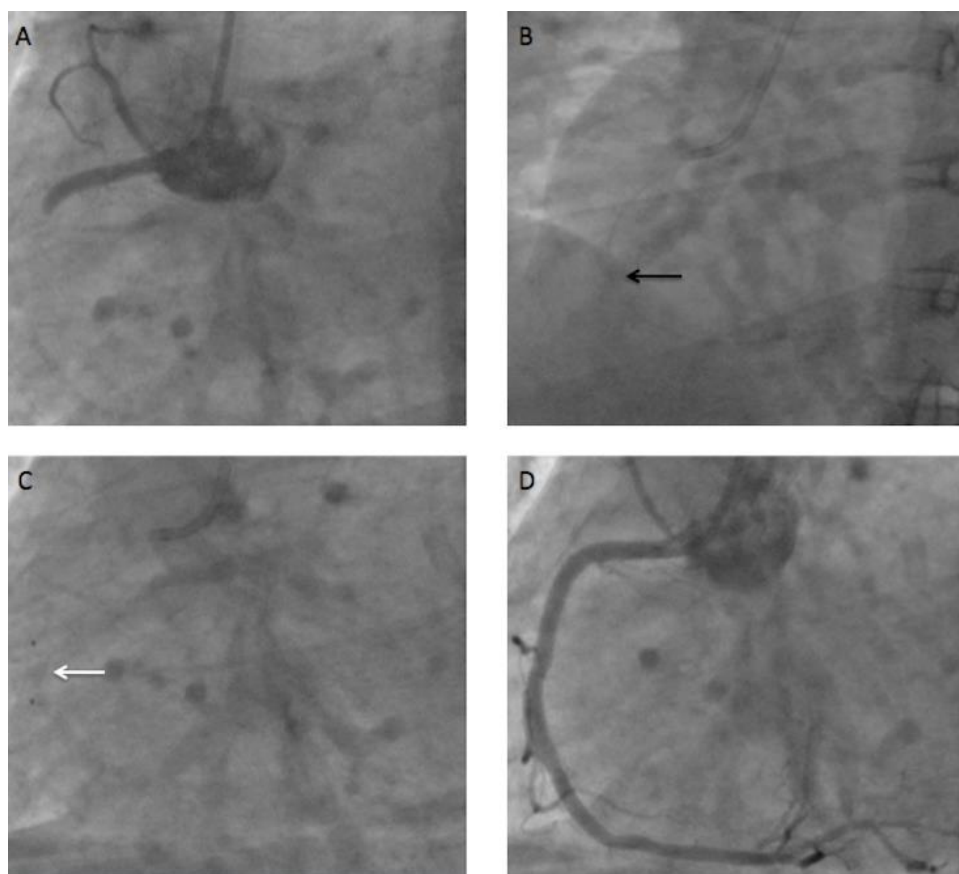
Figure 2.1. Example labels of study IMP

### 2.1.7 Percutaneous Coronary Intervention

After crossing the obstruction of the infarct-related coronary artery with a guide wire, an over-the-wire balloon (Emerge, Boston Scientific, Natick, MA, USA) was positioned beyond the obstruction (Figure 2.2). The guide wire was removed and the study drug solution injected by hand through the central lumen of the balloon catheter into the distal vascular bed over 30 seconds. The guide wire was then reinserted through the balloon catheter and advanced to a distal position. The procedure was then continued as per operator preference with no restriction placed on vascular access route, type of stent or method of stenting (pre-dilatation or direct). Patients undergoing non-culprit PCI were excluded from the



trial. Staging of non-culprit PCI was allowed.



**Figure 2.2. Delivery of investigational medicinal product**

Panel A: Fluoroscopic image demonstrating an occluded right coronary artery. Panel B: Fluoroscopic imaging showing a standard 0.014 guide-wire passed across the obstruction as depicted by the black arrow. Panel C: Fluoroscopic imaging displaying the position of the over-the-wire balloon (OTW) (white arrow) for the infusion of the IMP. Here the OTW balloon has been advanced over the wire, the wire removed and the IMP injected through OTW balloon over 30 seconds. Panel D: Fluoroscopic imaging displaying the end result of the procedure where the artery has been opened with stent implantation. Here the wire was re-advanced through the balloon, the balloon removed and the procedure continued as normal with balloon pre-dilatation and stent implantation.

## **2.1.8 Study Endpoints**

### **2.1.8.1 Primary endpoint**

The primary endpoint was myocardial infarct size, assessed by 48-hour area under the curve (AUC) plasma creatine kinase (CK). The area under the curve (AUC) (expressed in arbitrary units) for CK was measured in each patient by computerised planimetry (GraphPad prism version 5.0 for mac, San Diego, California). We chose to assess CK rather than CK-MB as this matches previous studies (Staat et al., 2005) and since CK measurement is part of the routine clinical assessments made in the UK following PCI. Moreover, whilst CK-MB might be considered more specific for cardiac injury recent evidence suggests that CK AUC is comparable to CK-MB, Troponin T or Troponin I for the assessment of infarct size (Chia et al., 2008).

### **2.1.8.2 Secondary endpoints-infarct size**

The principal secondary end point was the size of the infarct as measured by troponin T AUC and the area of delayed hyperenhancement evident by cardiac magnetic resonance imaging (CMR), assessed on day two and six months after infarction. The CMR scan was offered as a sub-study with separate consent to participants and therefore this estimation of infarct size was only performed in those patients consenting to the procedure. Additional CMR related secondary endpoints were left ventricular volumes, ejection fraction, and the myocardial salvage index.

#### **2.1.8.3 Secondary endpoints-Mechanistic**

Mechanistic endpoints included plasma nitrite and nitrate levels (collectively termed NO<sub>x</sub>) and plasma cGMP concentrations. These provide an indication of systemic NO levels and NO bioactivity (Ignarro et al., 1993). Markers of inflammation (flow cytometric assessment of circulating inflammatory cells and plasma levels of high-sensitivity C-reactive protein (hs-CRP), monocyte chemoattractant protein-1 (MCP-1), CXCL-1, and CXCL-5) and platelet reactivity (P-selectin expression and whole-blood aggregometry (WBA)) were measured at baseline, post procedure, at 4 hours, 24 hours and 6 months post-PPCI).

#### **2.1.8.4 Secondary endpoints-Safety**

Safety endpoints included the acute safety and tolerability of intra-coronary nitrite in STEMI (haemodynamics and in-patient major adverse events occurring after reperfusion, including death, heart failure, AMI, stroke, recurrent ischaemia, need for repeat revascularization, renal/hepatic insufficiency, vascular complications, and bleeding. Additional safety endpoints included an assessment of ventricular rhythm disturbance for 24 hours post primary PCI and an assessment of major adverse cardiac events (MACE) at 6 and 12 months (death, MI, stroke, heart failure, need for repeat revascularization (PCI or CABG). MI was defined as new ischaemic pain with new ST elevation or ECG changes, and further elevation of enzymes, whether treated with further revascularisation therapy or not), (PCI or emergent CABG). Stroke was defined according to World Health Organization criteria, which defines a stroke as a focal deficit that lasts for more

than 24 hours. Heart failure was defined as dyspnoea accompanied by both physical signs of heart failure (pulmonary crackles/rales, peripheral oedema, jugular venous distension, S3 gallop, radiological evidence of pulmonary oedema) and a need for increased heart failure therapy (diuretic or other oral heart failure therapies e.g. ACEi or mechanical/surgical intervention).

### **2.1.9 Blood sampling**

Blood samples were taken by standard venepuncture into pre-chilled vacutainer tubes containing 1.8mg EDTA (for NO<sub>x</sub>), 0.1 mmol/L 3-isobutyl-1-methylxanthine (IBMX: added to cGMP samples to prevent cleavage of cGMP) silica (separate tubes for CK/trop T, chemokine and N-terminal-pro-brain natriuretic peptide (BNP) analysis) or 3.2% buffered sodium citrate (for aggregation and flow cytometry analysis) (Becton, Dickinson & Co, Franklin Lakes, USA). NT-pro-BNP levels have been shown to identify patients post AMI who are at risk of LV dysfunction, heart failure, and death and is complementary to the measurement of LVEF (Omland et al., 1996; Richards et al., 1998). All samples aside from citrate containing tubes and samples sent to Barts Health laboratory were immediately centrifuged (Heraeus Megafuge 16R, Thermo Scientific, Karlsruhe, Germany) (1300g, 4°C, 10 min). Plasma was separated and stored at -80°C until measurement of NO<sub>x</sub> (see section 2.1.17) cGMP (section 2.1.16) or chemokine (section 2.1.15) levels were undertaken. Bloods taken for Urea and electrolytes, CK and troponin T analysis were sent to Dr Sally Benton, Consultant in Clinical Biochemistry, Department of Biochemistry, Barts Health NHS Trust and were processed as per standard clinical laboratory methods.

Samples taken for mean platelet volume (MPV) were sent to Jyoti Farren, Chief Biomedical Scientist, Department of Haematology, Barts Health NHS Trust and were processed as per standard clinical laboratory methods. MPV was measured on baseline samples for 60 patients and was measured as baseline levels have been linked to impaired angiographic reperfusion and mortality after primary PCI for STEMI (Huczek et al 2005). Samples for BNP were sent to the Doctors laboratory (60 Whitfield Street, London, W1T 4EU).

Blood samples for Troponin-T and CK were taken prior to primary PCI and at 4, 8, 12, 18, 24, 36 and 48 hours post-procedure. AUCs were measured over 48 instead of 72 hours as in other studies (Piot et al., 2008) as it was routine practice at the trial institution for patients to remain in hospital for 48 hours after a successful PPCI, unless there was a reason for a longer stay i.e. cardiogenic shock. Therefore the trial was designed to best fit with Barts Health Trust's clinical policies/pathways and neither delay the patient's discharge nor necessitate their return for further blood tests after discharge.

Arterial blood gases were taken pre and post-primary PCI to assess levels of methaemoglobin. This was performed to assess for one of the main potential side effects of nitrite infusion: methaemoglobinaemia. NT-pro BNP was measured at baseline, 48 hours and 6 months. Blood samples for markers of inflammation (CXCL-5, CXCL-1, MCP-1 and Hs-CRP), platelet reactivity (P-selectin expression, WBA), and nitrite/nitrate/cGMP levels were taken at baseline, 30 minutes, 4, 24 hours and 6 months post primary PCI.

### **2.1.10 CMR imaging**

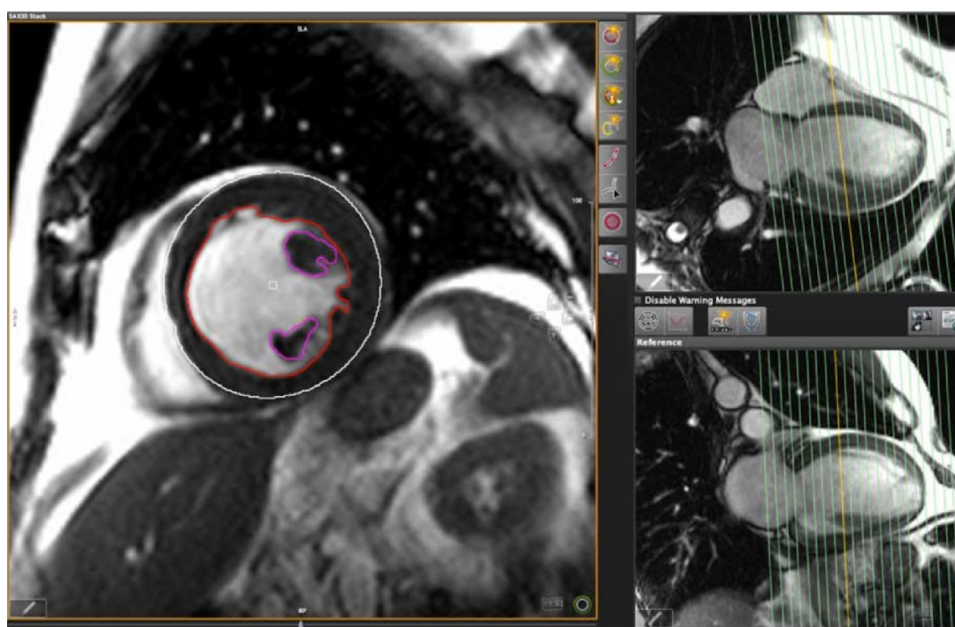
CMR imaging was performed on a 1.5 T Philips Achieva scanner with a cardiac 32-channel phased array coil. Each examination used cine-CMR for ventricular volumes and function, T2-weighted imaging for myocardial oedema and delayed enhancement CMR for infarct size assessment and evaluation of microvascular obstruction (MVO). Cine CMR is considered the gold standard of the evaluation of cardiac volumes, mass, and systolic function since it does not apply geometric assumptions and has excellent reproducibility and accuracy (standard error for left ventricular, LV, mass and volume is approximately 5%) (Grothues et al., 2004; Hundley et al., 2010). Balanced steady-state free precession cine imaging was used to acquire 10-12 short axis slices (8 mm slice thickness, 2mm gap) with one slice per breath-hold. Sequence parameters were 1.5 ms echo time (TE), 3.1 ms repetition time (TR), and acquired voxel size was 1.8 x 1.86mm with a typical field of view (FOV) of 360mm in the phase encode direction. 45 phases were acquired with 25% phase sharing. Parallel imaging (SENSE) was used with an acceleration factor of 2.0.

Delayed gadolinium enhancement (DE) images are T1-weighted inversion recovery sequences acquired about 10 minutes after the intravenous administration of gadolinium with an inversion time chosen to null the myocardial signal (Simonetti et al., 2001) using an 'inversion time scout' or 'look locker' sequence. Gadolinium is an extracellular agent, which enhances its distribution volume in certain conditions such as necrotic myocardium, assuming a bright signal (hyperenhancement), opposed to dark viable (normal)

myocardium (Judd et al., 1995). CMR highlights the region of scar as small as 0.16 g (Wu et al., 2001) and the reproducibility is high with a coefficient of reproducibility reported equal to +2.4% of LV mass in the chronic setting (Mahrholdt et al., 2002). Delayed enhancement images were acquired ten minutes after injection of a dose of 0.2 mmol/kg of gadoterate meglumine (Dotarem) for delayed gadolinium enhancement. A T1-weighted segmented inversion-recovery gradient echo pulse sequence (TR 3.9ms TE 1.9ms, flip angle 15°, voxel size of 2 x 2mm, typical FOV 360mm) was used to obtain 10-12 short axis slices (matched with short-axis cine images) with one slice per breath-hold. The inversion time was adjusted individually according to a T1 scout sequence (Look-Locker). Images were acquired every other heart beat with 2 signal averages.

Myocardial oedema is one of the earliest manifestations of ischaemia and occurs before the development of definitive and irreversible damage, T2-weighted imaging may be used to visualise the ischaemic area at risk (AAR) (Beek and van Rossum, 2010). Increased myocardial water content increases signal on T2-weighted images due to a prolongation of the T2 relaxation time (Higgins et al., 1983). Myocardial oedema in the acute phase of AMI can therefore be visualized as a bright signal on T2-weighted images, defining the 'myocardium or area at risk' (Aletras et al., 2006). Myocardial oedema was assessed using fat suppressed T2-weighted triple inversion turbo spin echo STIR (Short tau inversion recovery) imaging (TE 80ms, TR 2 heart beats, TSE factor 31, voxel size 1.8 x 1.8mm). 10-12 slices were obtained (8mm per slice, 2mm gap matched to DE/cine slices) with one slice per breath-hold.

Images were anonymised, batched and analyzed in blinded fashion by two experienced operators. Scar and oedema volumes were calculated by manually drawing endocardial and epicardial contours followed by semi-automated selection of normal remote myocardium per slice (Figure 2.3). Myocardial oedema was described as  $>2SD$  in signal intensity from remote normal myocardium. Infarct size was calculated using the full-width half maximum method as previously described (Flett et al., 2011). In case of discordance between operators, blinded review by a level III accredited CMR reader was performed (Dr Mark Westwood). Analysis was performed using dedicated software (CVI<sup>42</sup>, Circle Cardiovascular Imaging Inc, Calgary, Alberta, Canada). Interobserver variability was calculated.



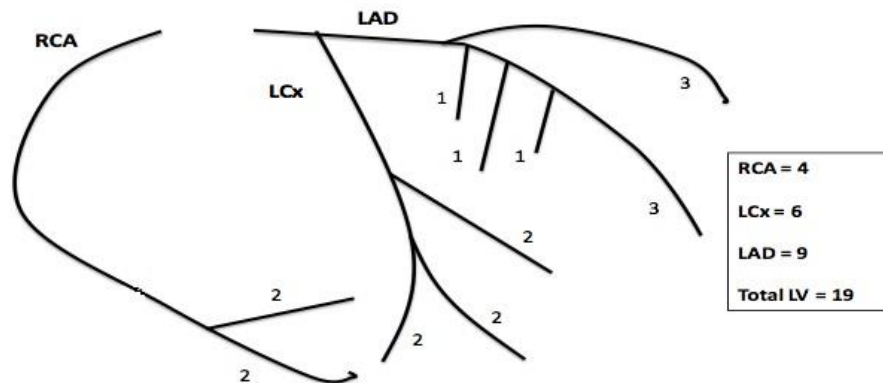
**Figure 2.3. LV function analysis in short axis images using long axis views as a cross-reference.**

Left panel: Diastolic mid-ventricular short axis view with contours. Right upper panel: Example long axis reference view in a 4-chamber orientation. Right lower panel: Example long axis reference view in a 2-chamber orientation. The orange lines represent the location of the cross-sectional long axis views used for measurements.



### 2.1.11 Determining the myocardial salvage index

When assessing the efficacy of a reperfusion treatment strategy, it is essential to express myocardial infarct size as a percentage of the area-at-risk (AAR). In this study, the AAR was quantified using both coronary angiography (modified Bypass Angioplasty Revascularisation Investigation [BARI] (Alderman and Stadius, 1992) (Figure 2.4) and the modified Alberta Provincial Project for Outcome Assessment in Coronary Heart Disease [APPROACH] (Ortiz-Perez et al., 2007) (Figure 2.5) jeopardy scores) and the acute (2 day) CMR scan (Myocardial oedema-T2 imaging). Myocardial salvage index was calculated from the CMR scan according to  $(\text{AAR} - \text{Infarct size}) / \text{AAR}$ .



RCA- right coronary artery, LCx- left circumflex artery, LAD- left anterior descending, LV- left ventricle.

**Figure 2.4** Diagram to show the BARI angiographic myocardial risk score (Alderman and Stadius, 1992)

Culprit lesion Location	Infarct related artery Side branches		Size		
			Diagonal (LAD)	Posterolateral (All others)	
			Small	Medium	Large
LAD (Right dominance or Left dominance)		Distal	13.75	14.8	15.9
		Mid	27.5	29.7	31.8
		Proximal	41.25	44.5	47.75
Proximal LCx (Right dominance)	Obtuse marginal	Small	9.25	12.5	15.75
		Medium	15.25	18.5	21.75
		Large	21.25	24.5	27.75
Proximal LCx (Left dominance)	Posterior descending artery (PDA)	Small	23.5	28	32.5
		Medium	29.5	34	38.5
		Large	35.5	40	44.5
RCA (Right dominance) Mid LCx (Left dominance)	Posterior descending artery (PDA)	Small	9.25	12.5	15.75
		Medium	15.25	18.5	21.75
		Large	21.25	24.5	27.75
Mid LCx (Right dominance)			3.25	6.5	9.75

**Figure 2.5 The modified- Alberta Provincial Project for Outcome Assessment in Coronary Heart Disease (APPROACH) angiographic myocardial risk score (Ortiz-Perez et al., 2007)**

### 2.1.12 Angiographic analysis

Coronary angiograms obtained before and after primary PCI were reviewed by two experienced observers blinded to treatment allocation and clinical data. From these angiograms an assessment of Thrombolysis In Myocardial Infarction (TIMI) flow grade (Table 2.1) and area at risk using both the modified BARI and modified APPROACH jeopardy scores were made. The TIMI flow grade was developed by TIMI study group for grading coronary blood flow based on visual assessment of the rate of contrast opacification of the infarct artery. The TIMI flow grade has become the standard for semi-quantitative evaluation of myocardial perfusion before and after coronary reperfusion therapies (TIMI Study Group, 1985) (TIMI Study Group, 1989).

TIMI 0 Flow = no penetration of contrast beyond stenosis (100% stenosis, occlusion)
TIMI 1 Flow = penetration of contrast beyond stenosis but no perfusion of distal vessel (99% stenosis, sub-total occlusion)
TIMI 2 Flow = contrast reaches the entire distal vessel but either at a decreased rate of filling or clearing versus the other coronary arteries (partial perfusion)
TIMI 3 Flow = contrast reaches the distal bed and clears at an equivalent rate versus the other coronary arteries (complete perfusion)

**Table 2.1. Thrombolysis in myocardial infarction (TIMI) flow grade classification**

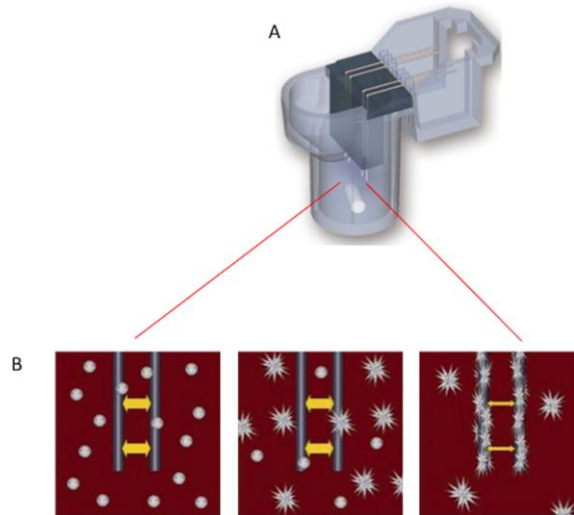
Syntax scores were calculated using <http://www.syntaxscore.com>. This is a scoring system used to quantify the complexity of coronary artery disease taking into account not only the number of significant lesions and their location but also the complexity of each lesion independently.

#### **2.1.12 Measurement of whole blood aggregometry**

Whole blood aggregation was determined using an impedance aggregometer (Multiplate® analyser, Dynabyte Medical, Munich). Impedance aggregometry is based on the principle that platelets are non-thrombogenic in their resting state, but expose receptors on their surface when they get activated which allow them to attach on vascular injuries and artificial surfaces. The device has five channels

for parallel tests, and a single-use test cell with duplicate impedance sensors, each consisting of two straight silver coated electrode wires (blood contacting area: 3 mm length, 0.3 mm diameter for each sensor wire) (Figure 2.6). When platelets stick on the Multiplate® sensor wires, they enhance the electrical resistance (impedance) between the two electrodes, which is continuously recorded (Figure 2.7). This delivers automated quality control of measurements by comparison of the two simultaneously recorded single analyses. The method has been described previously (Tóth et al., 2006).

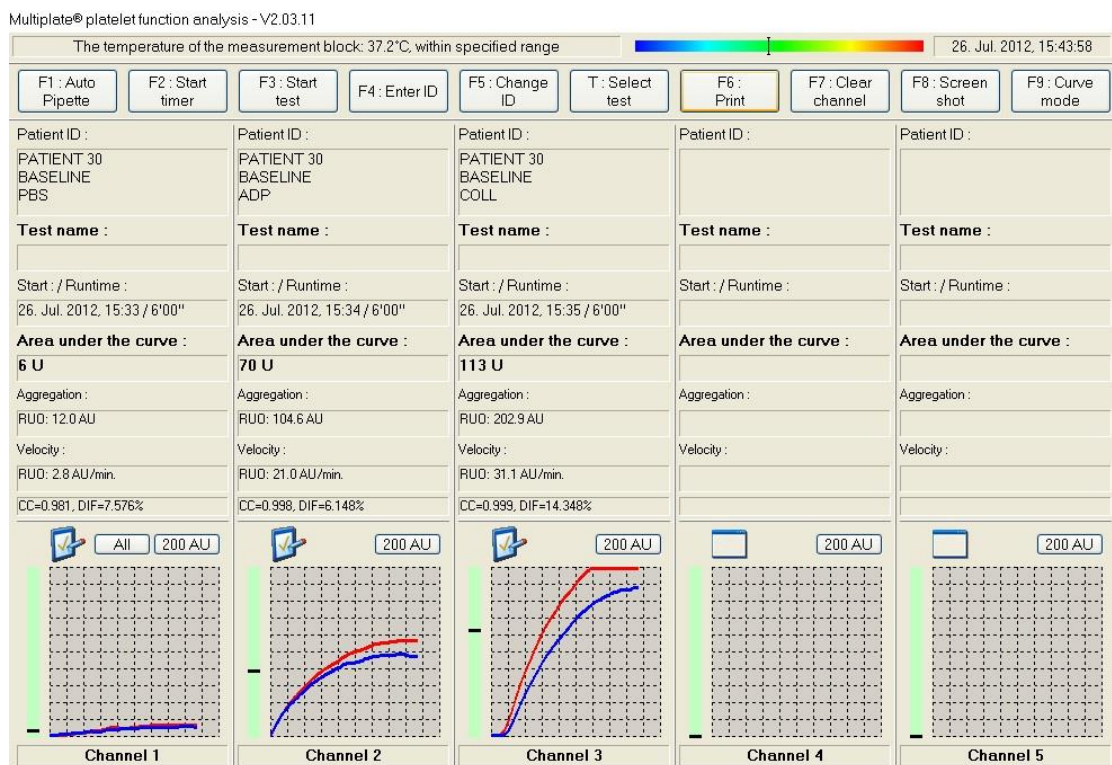
In the test cuvette, whole blood (300 µL) is diluted, 1:1, with 0.9% NaCL with 3 mmol/L CaCL<sub>2</sub> (Sigma, UK) solution maintained at 37°C for 3 minutes. At the end of this time, 12µl of agonist was added to give end concentrations of ADP of 10µM (ChronoLog, Havertown, PA) and 3µg/ml of collagen (Nycomed, Munich, Germany). Phosphate buffered saline (PBS) was added to one test-cell as a control. The increase of impedance due to the attachment of platelets to the electrodes was detected for each sensor unit separately and transformed to arbitrary aggregation units (AU) that were plotted against time (AU\*min). The aggregation measured with this device was quantified as area under the curve (AUC) of AU\*min. (Figure 2.7)



**Figure 2.6. How the multiplate cell works**

A. Disposable test cell with duplicate sensor electrodes

B. Activated platelets adhere on the surfaces of the sensor. The impedance between the electrodes rises as the adhered platelet aggregates form an insulation layer

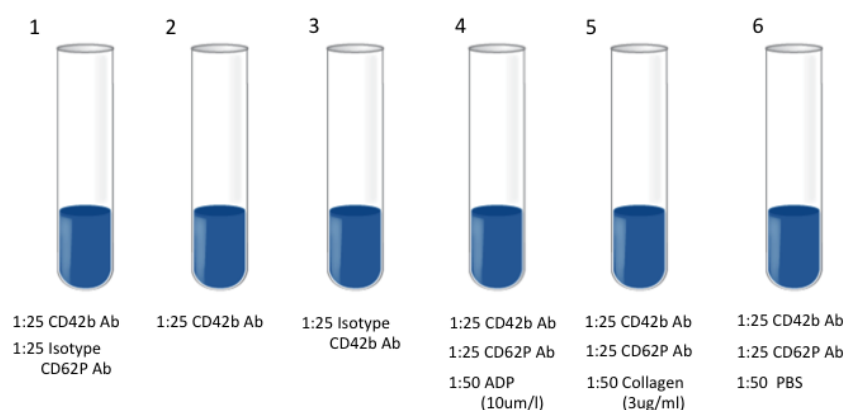


**Figure 2.7. Screenshot depicting AUC measurement for Whole-blood aggregometry**

### 2.1.13 Measurement of Platelet P-selectin expression

Two-colour whole blood flow cytometry was used to measure platelet P-selectin using a previously published protocol (Janes et al., 1993). Whole blood was collected from individuals at the specified time-points (baseline, 30 minutes, 4 hours, 24 hours and 6 months post IMP).

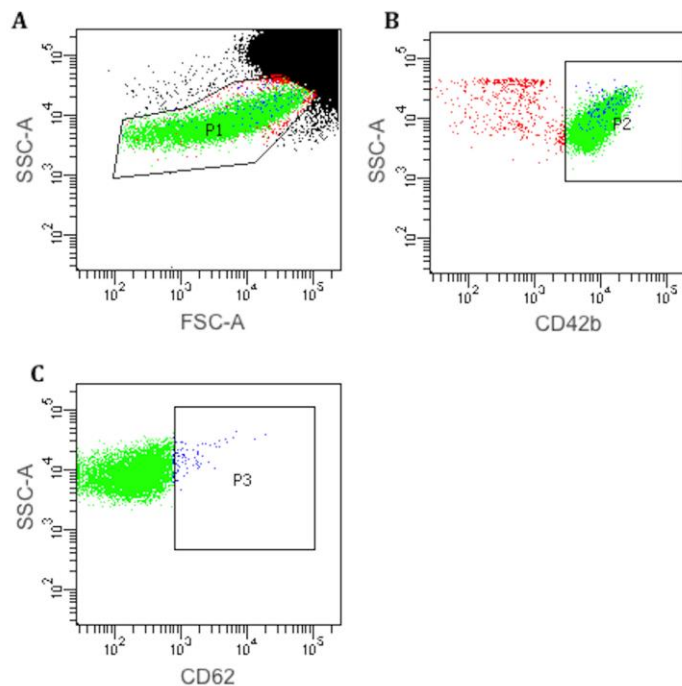
Five  $\mu\text{L}$  of citrated blood was immediately added to 45 $\mu\text{L}$  of HEPES-buffered saline containing appropriate concentrations of antibodies (isotype control of IgG or IgM; or platelet markers CD42b and CD62P) and agonists: ADP 10  $\mu\text{mol/L}$ , collagen 3 $\mu\text{g/ml}$  or PBS as a control sample (Figure 2.8). All samples were run in duplicate. After gentle mixing, the samples were incubated at room temperature for 20 minutes, and then fixed using 1% paraformaldehyde (Sigma, UK) stored at 4°C and then analysed using flow cytometry within 48 hours.



**Figure 2.8 Sample make-up for P-Selectin expression**

#### **2.1.13.1 Flow cytometry**

Flow cytometry is a widely used technique, which enables detailed quantitative information to be gathered on cells in a large cell population. Samples were acquired and recorded on the LSR Fortessa II Cell Analyzer (BD Biosciences, San Jose, CA, USA) using BD FACSDiva Software (BD Biosciences, San Jose, CA, USA). The flow cytometer analyses deflected light from each individual cell, which is recorded as forward-scatter (FSC; proportional to the size of the cell) and side-scatter (SSC; relative to granularity and internal complexity of the cell) on a FSC/SSC dot-plot graph. Fluorochromes conjugated to antibodies bind to specific cell surface markers and the presence of these markers can be quantified by measuring the fluorescence emission. For the assessment of the platelet population in blood samples, platelets were first identified preliminarily based on their forward and side-scatter properties, then further delineated via labeling with CD42b monoclonal antibody conjugated to allophycocyanin (APC) (Biolegend, San Diego, USA) Gates were used to isolate this population, and CD62 (P-selectin) (Serotec Canada Ltd) monoclonal antibody conjugated to fluorescein-isothiocyanate (FITC) was used to determine P-selectin expression. Selectivity of populations were further confirmed by use of antibody negative iso-types to P-selectin and CD42b (Biolegend, San Diego, USA). 10,000 platelets were acquired in the CD42b region, and results were expressed as the percentage of platelets positive for P-selectin (Figure 2.9).



**Figure 2.9. Flow cytometry assessment of P-selectin expression**

- A. Platelets were first identified based on their forward and side-scatter properties (P1)
- B. Then further delineated via labelling with CD42b monoclonal antibody conjugated to allophycocyanin (APC) (P2)
- C. CD62 (P-selectin) monoclonal antibody was used to determine P-selectin expression (P3)

#### **2.1.14 Measurement of circulating cell populations and their activation state**

The systemic inflammatory response post myocardial infarction was assessed using monoclonal antibodies conjugated to fluorochromes to identify lymphocytes, monocytes and neutrophils in the systemic circulation followed by the expression of CD162, CD62L and CD11b on their surfaces as indices of cell activation (see table 2.2).



#### **2.1.14.1 Preparation of samples for flow cytometry**

A total of 50µL of citrated blood was added to tubes containing specific antibodies (CD14, CD16, CD16b, CD3, CD4, CD8, CD11b, CD62L, CD162 and isotype controls for CD11b, CD162, and CD62L, see table 2.2 for dilutions), mixed gently and then allowed to incubate for 30 minutes. Excess antibody was washed off by adding 2ml of PBS to each tube and centrifuged for 5 minutes, 400g at 4°C. After decanting the supernatant, 500µL of a working solution of a whole blood lysing reagent (Beckman Coulter Inc.) was added to each tube. The samples were washed twice using 2mL of PBS and centrifuged for 5 minutes, 400g at 21°C. To the supernatant 500µL of 1% paraformaldehyde (Sigma, UK) was added to the tubes. All samples were then analysed using BD LSR Fortessa Cell Analyser (BD; flow cytometer) and recorded with BD FACSDiva software.

#### **2.1.14.2. Flow cytometry and analysis**

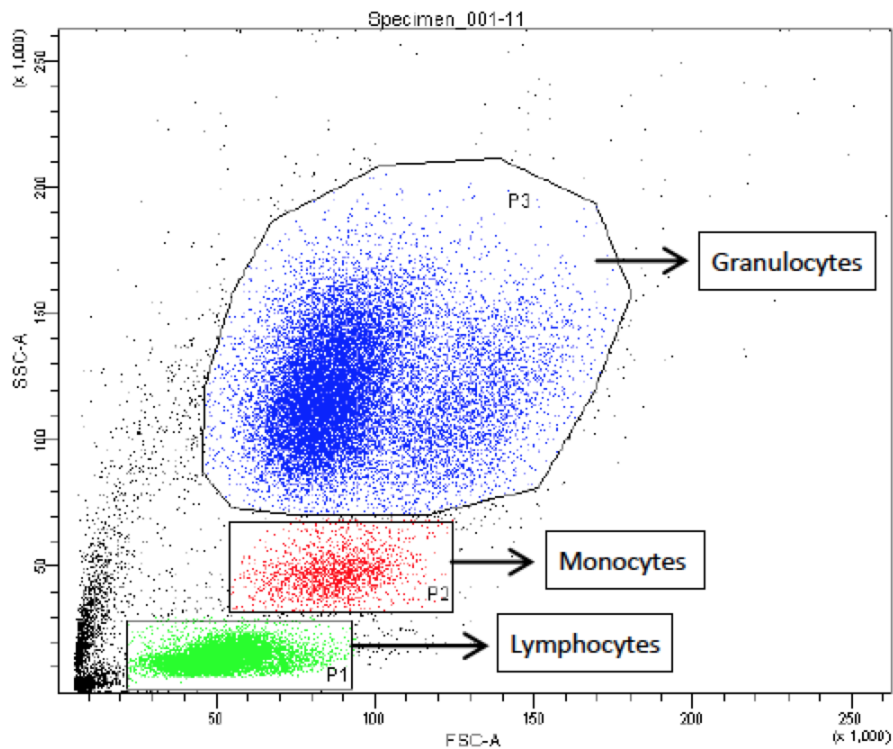
The principles of flow cytometry were described in section 2.1.13.1 and the same principles apply to the analysis of the cell types and activation markers. Combined with FSC and SSC data used to identify cell types (Figure 2.10) the staining pattern of each leukocyte was used to calculate the relative percentage of cells expressing each inflammatory marker. In addition, in each case the respective median fluorescence intensity (MFI) was determined and provides a measure of the number of markers present on each cell type. To ensure that the fluorescence recorded was due to specific primary antibody binding to the desired marker, and not due to non specific binding (i.e. Fc receptors), isotype-

<b><u>Antigen</u></b>	<b><u>Role Of Surface Marker</u></b>	<b><u>Cells/inflammatory marker identified using antibody</u></b>	<b><u>Fluorochrome Conjugated With Antibody</u></b>	<b><u>Source</u></b>	<b><u>Dilution</u></b>
CD14	Pattern recognition receptor which binds bacterial lipopolysaccharide	Monocytes	Fluorescein isothiocyanate (FITC)	Beckton, Dickinson & company, New Jersey, USA	20µL
CD16	Low affinity Fc receptor found on natural killer cells, neutrophils and monocytes	Monocytes, neutrophils	Phycoerythrin (PE)	Beckton, Dickinson & company, New Jersey, USA	2 µL
CD16b	Fc receptor (FcγRIIIb) which binds to Fc portion of IgG antibodies	Neutrophils (Granulocytes)	PE	Beckton, Dickinson & company, New Jersey, USA	20µL
CD3	Forms part of the T-cell co-receptor required for T-cell activation	Lymphocytes	FITC	eBioscience Inc, San Diego, USA	2.5µL
CD4	Glycoprotein expressed on T-helper cells which interact with MHC-II molecules	CD4+ Lymphocytes	Combined PE & cyanine dye (Cy7 (PE-Cy7)	eBioscience Inc, San Diego, USA	2.5µL
CD8	Glycoprotein expressed on cytotoxic T-cells which interact with MHC-I molecules	CD8+ Lymphocytes	Combined peridinin chlorophyll protein & cyanine dye (PerCP-Cy5.5)	eBioscience Inc, San Diego, USA	2.5µL
CD162	Expressed by activated leukocytes, PSGL-1 is the high affinity receptor for P-selectin (expressed by platelets and endothelial cells, important for leukocyte rolling)	P-Selectin Glycoprotein Ligand-1	Allophycocyanin (APC)	eBioscience Inc, San Diego, USA	5µL
CD62L	Expressed by activated macrophages, monocytes and neutrophils. Important for leukocyte rolling	L-Selectin	APC-eFluor 780	eBioscience Inc, San Diego, USA	5µL
CD11b	Expressed by activated leukocytes. Aids in leukocyte adhesion and migration	Integrin alpha M (ITGAM) or Macrophage-1 antigen (MAC-1)	Pacific blue (PB)	Beckton, Dickinson & company, New Jersey, USA	10µL

**Table 2.2. The fluorochrome conjugated antibodies used to identify specific cell surface markers**

matched control antibodies were used. MFIs were recorded in absolute values

Flow cytometry data were analysed using the BD FACSDiva 6.0 software. During flow cytometry the fluorochromes are excited by light, which causes the emission of fluorescent light at a longer wavelength. The fluorochromes do not emit light at a specific wavelength but over a fairly wide range known as emission spectra. Overlapping of emission spectra between fluorochromes can occur therefore when using multiple fluorochromes, the emitted light from the leading and trailing tails from a neighbouring fluorochrome is registered.

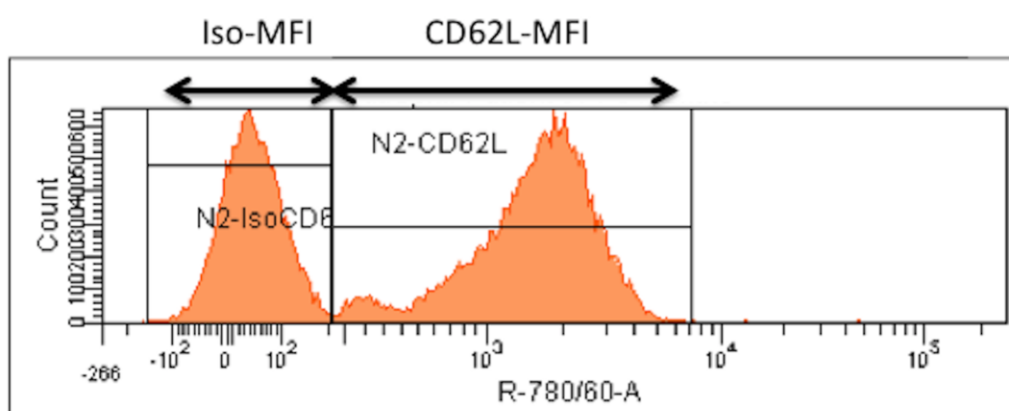


**Figure 2.10. Dot plot showing the FSC vs. SSC characteristics of leukocyte subpopulations derived from human blood.**

Lymphocytes are the smallest leukocytes with little granularity in the cytoplasm. They are identified by having a low FSC and SSC (P1 gate; green). Monocytes (P2 gate; red) are larger in size and therefore distinguished by a higher FSC. Granulocytes (predominantly neutrophils) are the largest cell type according to size and contain many granules within their cytoplasm and therefore have a higher FSC and SSC (P3 gate; blue). The black dots with low FSC and SSC represent dead cells and/or debris.

Since 7 different fluorochromes were used for cell type and activation marker analysis, spectral overlap had to be accounted for by manual compensation to allow accurate data analysis. In order to manually compensate, single staining of samples with each antibody conjugated fluorochrome was recorded on the flow cytometer.

Analysis of cell population size and fluorescent intensities of the different leukocytes were achieved by constructing dot plots and histograms. In order to segregate leukocyte populations accurately, gates had to be established on the dot-plots. The quadrant boundaries between cells that stained positive and negative for a particular leukocyte population was determined according to the fluorescence distribution of positively stained cell relative to the unstained sample. MFIs were reported as ratios relative to the MFI of the iso-control antibodies. (Figure 2.11)



**Figure 2.11: Calculation of the relative Fluorescence Intensity (RFI)**

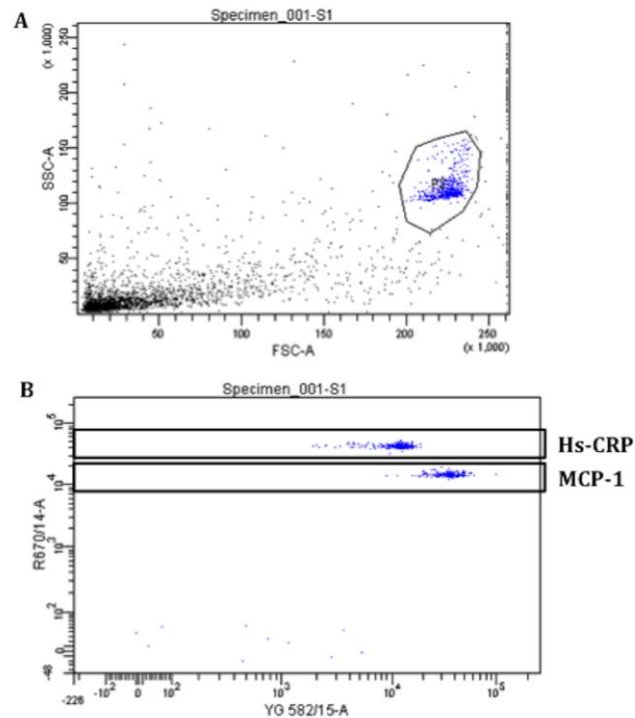
Example histograms staining positive for CD62L and the isotype control for CD62L. Panel shows the MFI for the CD62L iso-type control and a histogram of cells positively stained for CD62L. The relative fluorescence intensity (RFI) was calculated by dividing the MFI for the positive antibody by the MFI for the iso-type control for each marker.

## **2.1.15 Measurement of circulating inflammatory mediators**

### **2.1.15.1 MCP-1/CRP (Flowcytomix)**

Levels of the chemokine MCP-1 and Hs-CRP were determined by flow cytometry. A FlowCytomix five-plex-assay (eBioscience, Vienna, Austria) was performed. Each FlowCytomix contains antibodies carrying beads of different sizes that bind free mediator. These bead complexes are then labelled with a fluorescent dye and are then detected by a flow cytometer.

For each mediator 25µl of each standard (S1-7: Concentration range for MCP-1: 41-30000pg/ml; hs-CRP:0.1-70ng/ml), blank assay buffer and serum samples were transferred into flow cytometry tubes and mixed with 25µl of bead mixture and 50µl biotin-conjugate-mix. The tubes were then incubated for 2 hours at room temperature. The samples were then washed twice using 1ml of assay buffer. Following this 50µl of phycoerythrin (PE) conjugated streptavidin solution was added to the samples and further incubated for 1 hour at room temperature. Samples were then washed twice and 400µl of assay buffer added to the tubes. Sample data were then acquired by multiparameter flow cytometric analysis using a BD LSR Fortessa Cell Analyser (BD; flow cytometer) and recorded with BD FACSDiva software (Figure 2.12). The detection limit of the assay was 18.2 pg/mL and 0.067 ng/mL for MCP-1 and hs-CRP respectively. The mean inter-assay and intra-assay coefficients of variation for MCP-1 were 12.5% and 3.4% respectively whereas for hs-CRP the inter-assay coefficient variation was 3.4% and the intra-assay 9.6%.



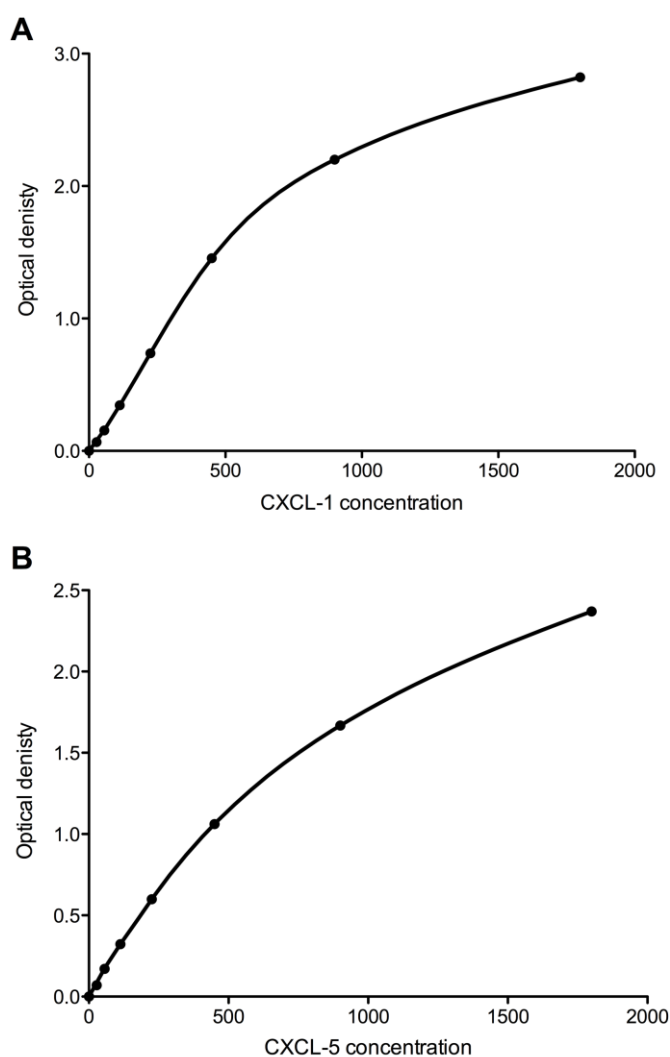
**Figure 2.12 Flowcytomix Flow cytometry**

The antibody bead population (P1) was gated according to the FSC/SSC and the variable signal from the 700nm excitation probe (R670/14-A). This allows differentiation between MCP-1 and hs-CRP (B). The amount of cytokines for numerous data-points vary in the amount of reporter fluorescence PE (YG 582/15-A). This allows standard curves to be generated from the MFI of the standards (S1-7). Test sample MFI values are then read off the standard curves.

#### **2.1.15.2 CXCL-1/CXCL-5 (ELISA)**

Circulating serum CXCL-5 (ENA-78) or CXCL-1 chemokine levels were assessed using 100µl frozen serum per direct sandwich ELISA in duplicate using the Human CXCL-5/ENA-78 DuoSet kit DY254 or the Human CXCL-1/Gro alpha duoset kit DY275 (R&D Systems, USA). In each case, a standard curve was generated (Figure 2.13) with the provided standards and utilized to calculate the quantity of chemokine in the sample tested. Ninety-six-well plates (R&D systems, USA) were coated overnight with capture antibodies at room temperature. After blocking of plates, supernatant samples and standard proteins were added to the

wells. After a 2 hour incubation period and a washing step, a biotin-labelled antibody was added to each well and incubated for an additional 2 hours. Plates were washed and streptavidin-horseradish peroxidase was added. Colour reaction was achieved using a tetramethylbenzidine substrate solution (TMB; and was stopped by a 1N sulphuric acid stop solution. Immediately thereafter, optical density values were measured at 450 and 570 nm on a spectrophotometric plate reader (MRX-TC Revelation, Dynex Technologies, UK). Optical values were corrected for the 96 well plate, by subtracting the 570nm values from the 470nm.



**Figure 2.13. Example standard curves for CXCL-1 and CXCL-5**

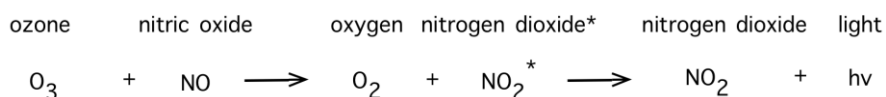
### **2.1.16 Measurement of plasma nitrite and nitrate concentration**

To measure plasma nitrite and nitrate concentration the technique of ozone chemiluminescence; a powerful quantitative analytical technique for measuring NO species in biological fluids, was used as previously described (Ignarro et al., 1993). Prior to chemiluminescence, plasma samples were filtered to remove proteins by centrifugation at 15000g for 60 minutes at 4°C, using Microcon® Ultracel YM\3 (3 kDa) (Millipore Corporation, Billerica, USA) filters that had been double-washed in NO<sub>x</sub> free water to remove any potential nitrate/nitrite contamination.

The chemiluminescence apparatus consists of two distinct components the purge vessel (reaction chamber) and the NO chemiluminescence analyser. Within the purge vessel, gaseous N<sub>2</sub> is continuously bubbled to render the chamber anoxic. Here, standards and biological samples containing NO<sub>x</sub> are reduced to NO in an equimolar fashion. The NO produced by these reactions is carried in the gaseous phase through the vessel into the NO chemiluminescence analyser (NOA 280i, Sievers, Manchester, UK), where it reacts with ozone. Light is emitted as a result of the chemical reaction (Equation 2.3), and is detected by the analyser, producing a digital signal corresponding to NO concentration (therefore NO<sub>x</sub> levels as appropriate).

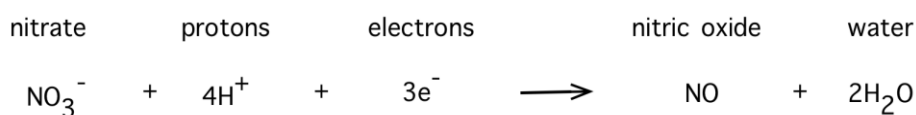
NO<sub>x</sub> levels were calculated by comparison to a standard curve generated daily from known standards, concentration range of sodium nitrite (0.1-10µM) and sodium nitrate (1-100µM) (see figure 2.14 and 2.15).



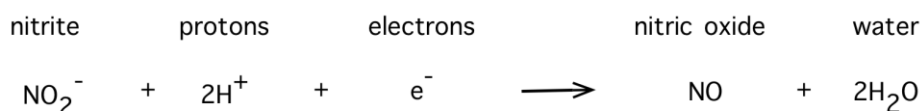


**Equation 2.3. Reaction of ozone and NO to chemiluminescent light (\*=nitrogen dioxide in an excited state)**

To determine total [NO<sub>x</sub>], samples were incubated in a strongly reducing environment using 0.1 mol/L vanadium (III) chloride in 1M hydrochloric acid refluxing at 95°C under N<sub>2</sub> which results in a sequential reduction of all NO<sub>3</sub><sup>-</sup> to NO<sub>2</sub><sup>-</sup>, and then all NO<sub>2</sub><sup>-</sup> to NO (Equations 2.4-2.5)

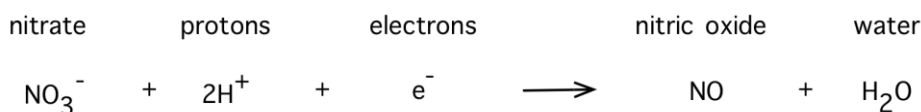


**Equation 2.4 Reduction of NO<sub>3</sub><sup>-</sup> by vanadium (III) chloride to produce NO.**



**Equation 2.5 Reduction of NO<sub>2</sub><sup>-</sup> by vanadium (III) chloride to produce NO.**

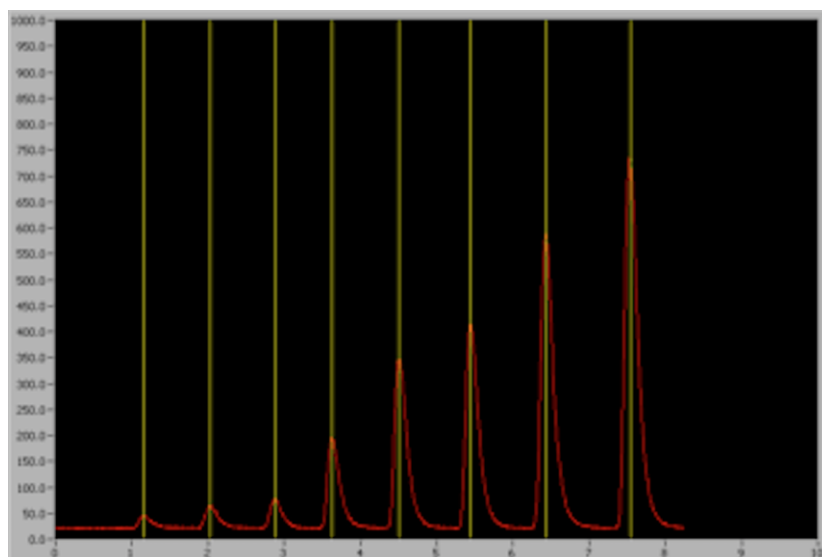
Concentration of nitrite from samples was determined by addition of samples to milder reducing conditions, 1.5 % potassium iodide in glacial acetic acid under N<sub>2</sub> at room temperature (equation 2.6), which is unable to reduce nitrate.



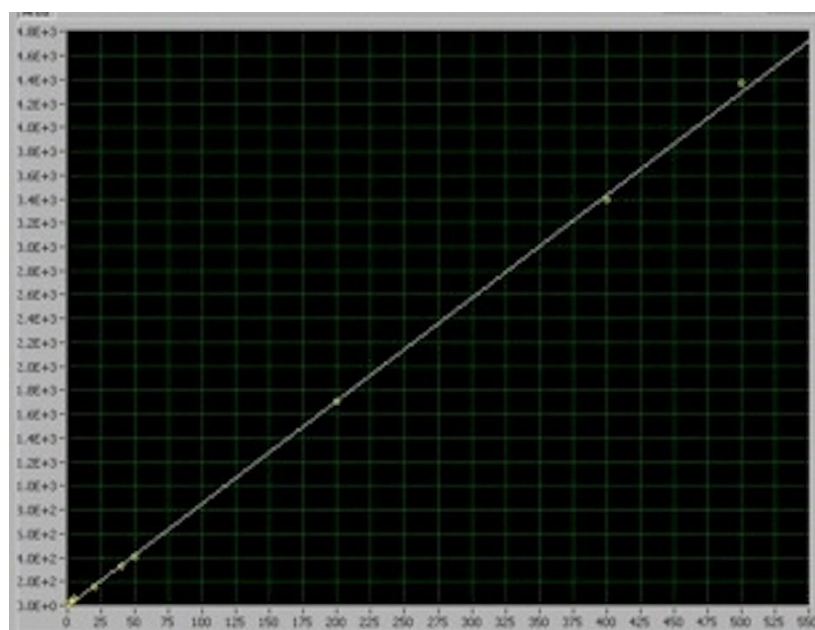
**Equation 2.6 Reduction of NO<sub>2</sub><sup>-</sup> by potassium iodide to produce NO.**

Concentrations of nitrate were calculated by subtraction of nitrite from NO<sub>x</sub>

values. Nitrate/nitrite concentrations were calculated by comparison to the standard curves.



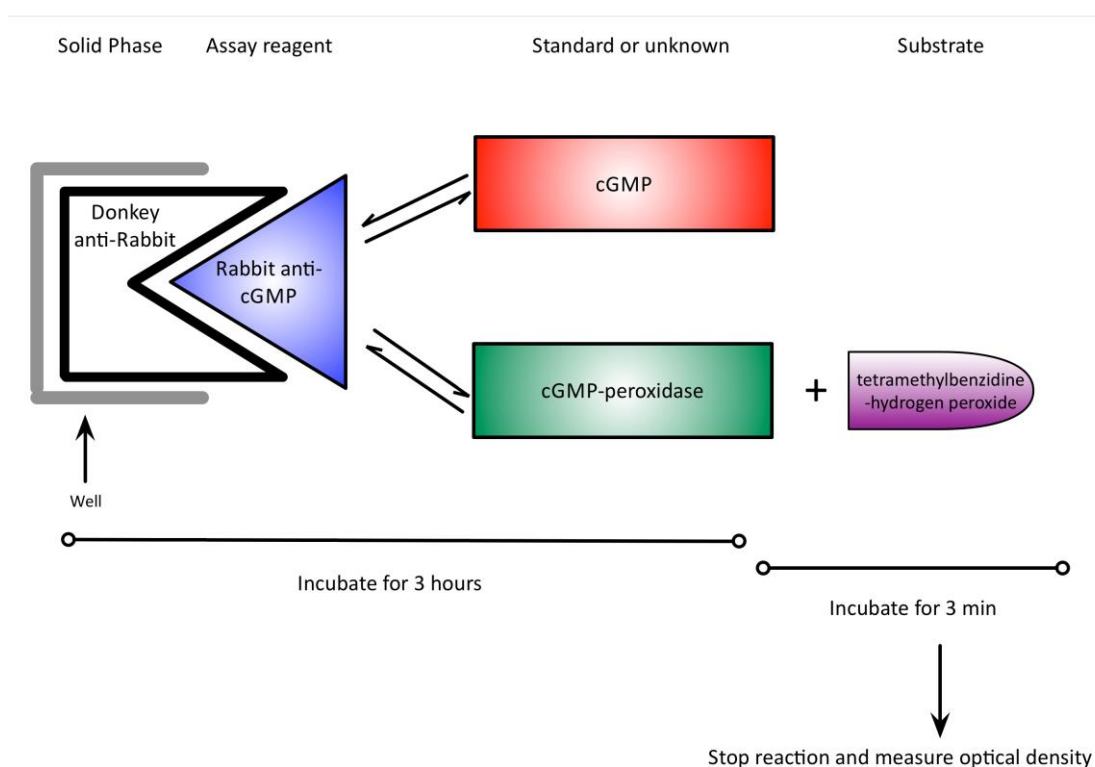
**Figure 2.14** An example trace that is produced when standards of known concentration are injected into the purge vessel.



**Figure 2.15** A typical standard curve produced prior to the measurement of nitrite in liquid samples (slope=8.6; intercept=-8.4;  $R^2=0.9996$ )

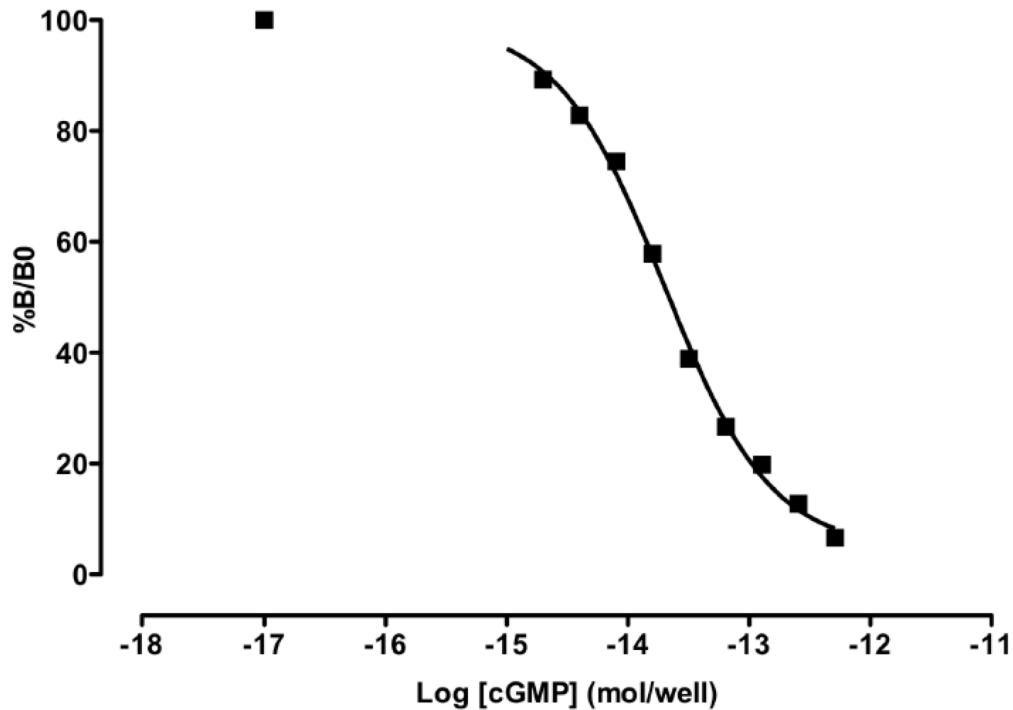
### 2.1.17 Measurement of plasma cGMP

On collection of plasma for cGMP determination, samples were incubated with a competitive non-selective phosphodiesterase (PDE) inhibitor, 3-isobutyl-1-methylxanthine (IBMX, 100 $\mu$ m) to prevent cleavage of cGMP during storage at -80°C. The concentration of cGMP in samples was determined using an enzyme immunoassay (cGMP Enzymeimmunoassay Biotrak System RPN226, GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions ([www.gelifesciences.com](http://www.gelifesciences.com)). The assay is based on competition between unlabeled cGMP and a fixed quantity of peroxidase labeled cGMP for binding sites on a cGMP-specific antibody (Figure 2.16). Samples measured were compared to a standard curve generated from known standards (Figure 2.17).



**Figure 2.16** Schema of enzyme immunoassay for measurement of cGMP levels  
(cGMP=cyclic Guanosine monophosphate)

Samples were diluted 1:20 in assay buffer (0.05M sodium acetate buffer pH 6.0 containing 0.002% (w/v) bovine serum albumin and 0.01% (w/v) preservative). Using a 96-well plate, 100µl of cGMP antibody was added to each well that had been pre-coated with donkey anti-rabbit IgG. To each well 20µl of acetylation reagent (2:1 of triethylamine and acetic acid) was added with 50µl of each sample or standard. Following a 2-hour incubation at 4°C, 100µl of lyophilized cGMP conjugated to horseradish peroxidase was added to each well and incubated at 4°C for 1 hour. Each well was then washed 4 times with wash buffer (0.01 M phosphate buffer pH 7.5 containing 0.05% (v/v) Tween™ 20) and 200µl of enzyme substrate 3,3',5'5'- tetramethylbenzidine (TMB) was added to each well and incubated at room temperature for 30 minutes. The colourless substrate TMB is converted into blue by the peroxidase and then 100µl of 1M sulphuric acid was added, turning the blue colour into yellow, and the plate was read at 450nm using a spectrophotometric plate reader (MRX-TC Revelation, Dynex Technologies, UK). The colour change depends on the amount of enzyme linked to the well and is inversely proportional to the cGMP concentration. Each sample and standard were analysed in duplicate.



**Figure 2.17 Standard curve for determination of cGMP levels.**

(%B/B0=% bound; cGMP=cyclic guanosine monophosphate).

### 2.1.18. Data and statistical analyses

Analysis was performed using GraphPad™ Prism software version 5.0 for Mac OsX and SPSS version 19, (SPSS Inc, Chicago, Ill). All p values are 2 sided and significance accepted as  $p < 0.05$ . Power calculations were performed using Graphpad statmate (Graphpad Software) and are represented in the experimental protocols in each chapter. For the clinical trial analysis was based on the intention-to-treat principle. Baseline demographic and clinical variables were summarised for each arm of the study. Descriptive summaries of the distributions of continuous baseline variables are presented in terms of percentiles (e.g. median, 25th and 75th percentile), while discrete variables are summarised in terms of frequencies and percentages. Comparisons are between

the sodium nitrite-treated and placebo control-treated group for the primary and secondary outcomes. Statistical analyses were conducted blind to the treatment groups. The statistical comparisons of the treatment arms with respect to the primary endpoint was performed using the Wilcoxon rank-sum test. For comparisons between normally distributed data statistical comparisons were performed using unpaired T test, for parametric data either the Mann-Whitney or Wilcoxon rank sum tests were used.

For comparisons between treatment groups assessing platelet reactivity, white cell counts and chemokine levels are expressed as mean  $\pm$  SEM and analysis was performed using two-way repeated measures ANOVA, including Bonferroni adjustment for multiple comparisons as appropriate.

Determination of correlations were performed using the Pearson's correlation coefficient analysis of least-squares and are expressed as 95% confidence intervals.

## **2.2 Cell culture experiments**

### **2.2.1 Human Endothelial Cell Culture**

Human coronary artery endothelial cells (HCAECs, Lonza, UK) and human umbilical vein endothelial cells (HUVECs, Lonza, UK) were cultured in endothelial growth medium (HCAECs: EGM-2 MV, HUVEC: EGM-2, Lonza, UK) containing endothelial growth factors, 5% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37°C in humidified air (5% CO<sub>2</sub>). Cells passaged 3 times to 80%-90% confluency were used (Figure 2.16).

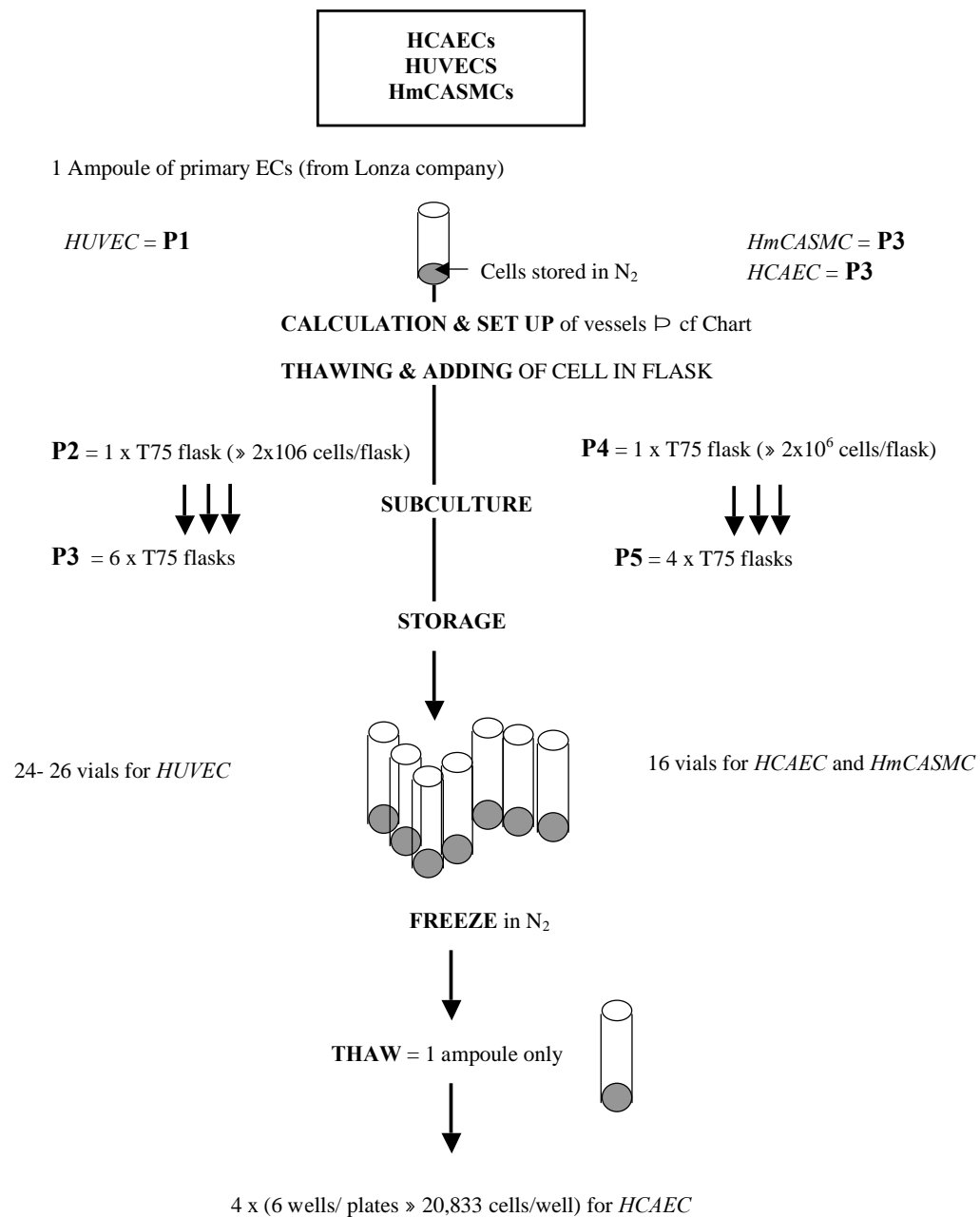
### **2.2.2 Human Coronary Artery Smooth Muscle cell culture**

Human Aortic Smooth muscle cells (HmCASMcs, Lonza, UK) were cultured in smooth muscle growth media (SmGM-2, Lonza, UK) containing smooth muscle cell growth factors, 5% fetal bovine serum (FBS), amphotericin and streptomycin at 37°C in humidified air (5% CO<sub>2</sub>). Cells passaged 3 times each to 80-90% confluency were used (Figure 2.18).

### **2.2.3 Cell Treatments**

#### **2.2.3.1 Hypoxia**

For hypoxia, confluent HCAECs/HUVECs and HmCASMcs were initially treated with various concentrations of sodium nitrite (0.1-10µM) and



**Figure 2.16. Diagram depicting method of cell passaging ready for experimentation**



then exposed to hypoxia (5% or 1% O<sub>2</sub>) by incubation in a Coy hypoxic glove box, (Coy Laboratory Products Ltd, Figure 2.19) at 37°C that subjected the cells to a controlled gas mixture consisting of N<sub>2</sub> and 5% CO<sub>2</sub> for 24 hours. For normoxic controls, cells were incubated in the standard CO<sub>2</sub> incubator under normoxic conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>, and 74% N<sub>2</sub>).



**Figure 2.19. Coy hypoxic glove box**

#### **2.2.3.2 I/R injury experiments**

For the I/R injury simulation studies, 70% confluent HCAECs and HmCASMCS were exposed to anoxia (0.1% O<sub>2</sub>) by incubation in a Coy Hypoxic Glove Box, (Coy laboratory products Ltd) at 37°C that subjected the cells to a controlled gas mixture consisting of N<sub>2</sub> and 5% CO<sub>2</sub> for 24 h. Cells were then reoxygenated by being returned to the standard CO<sub>2</sub> incubator under normoxic conditions (21% O<sub>2</sub>, 5% CO<sub>2</sub>, and 74% N<sub>2</sub>) with the replacement of fresh oxygenated EBM media

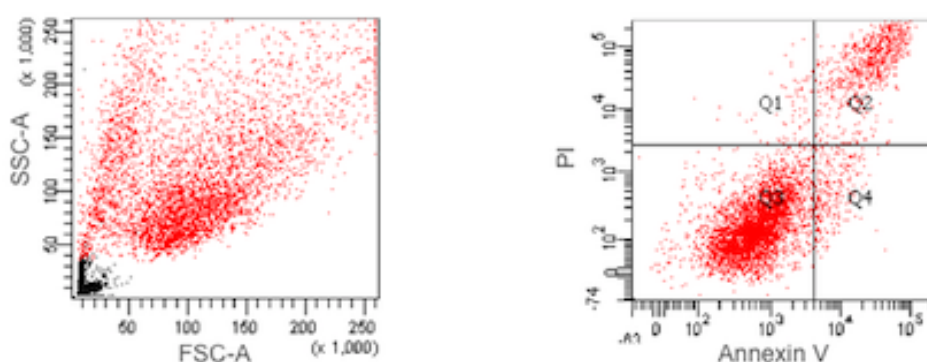
(to simulate reperfusion) or oxygenated EBM media containing nitrite (1  $\mu$ M or 3  $\mu$ M). Cells were then analysed at 8, 24h and 48h after reperfusion. For normoxic controls, cells were kept at 21% O<sub>2</sub> in the standard CO<sub>2</sub> incubator (5% CO<sub>2</sub>, and 74% N<sub>2</sub>) for 24h and then the EBM media replaced as above.

#### **2.2.4 Flow cytometry analyses of cell apoptosis and death**

Flow cytometry is a validated and effective method of identifying apoptosis with comprehensive reviews of its use, detailing descriptions of cell features that can be measured (Darzynkiewicz et al 1992; Vermes et al., 2000; Wlodkowic et al., 2012). During apoptosis phosphatidyl serine (PS) residues, which are normally located on the internal surface of the plasma membrane, redistribute to the external surface. PS binds the protein Annexin V and the change can be observed by incubating unfixed cells with fluorochrome labelled Annexin V. Combining detection using directly-conjugated Annexin V with viability exclusion using Propidium Iodide (PI) or 7-AAD (7-aminoactinomycin D) allows for assessment of non-apoptotic vs. early and late apoptotic cells. Viable cells will contain neither stain, apoptotic cells with intact plasma membrane are stained only by Annexin V-FITC, whereas cells in secondary necrosis, consecutive to apoptosis, contain both stains. This method is well established and has the advantage that Annexin V-FITC will bind immediately to cells which have surface exposed PS and can therefore be analysed almost instantaneously

Following treatment as described in section 2.2.3, HCAEC, HUVEC and HmCASM C cultures were washed with PBS, trypsinised and centrifuged at 1000 rpm for 5

min. The cell pellets were then re-suspended in PBS to remove any trace of trypsin and re-centrifuged at 700 g for 5 min. Cells were treated with recombinant annexin V antibody conjugated to Alexa Fluor 488 (Trevigen, Gaithersburg, USA) for determination of apoptosis. Necrotic cells were shown by binding of the red-fluorescence PI nucleic acid binding dye (Trevigen, Gaithersburg, USA). PI is impermeable to live cells and apoptotic cells, but stains necrotic cells with red fluorescence, binding tightly to the nucleic acid in the cell. All samples were then analysed using BD LSR Fortessa Cell Analyser (BD; flow cytometer) and recorded with BD FACSDiva software (Figure 2.20).



**Figure 2.20 HUVEC Viability assessed by flow cytometry under 5% hypoxia**

Representative flow cytometry dot plots of HUVECs exposed to 24 hours 5% hypoxia. Representative FSC versus SSC and the annexin V-FITC (B-530/30-A) versus propidium iodide (PI) (YG-710/50-A) plots are shown. Values in Q1 are positive for PI and negative for annexin and are necrotic cells. Q2 are cells positive for annexin V and PI and are apoptotic. Q3 are negative for both PI and annexin V (Viable), Q4 are early apoptotic (annexin positive and PI negative).

### 2.2.5 Cell counting

A haemocytometer consists of a special glass slide with an accurately ruled etched grid of precise dimensions. Originally developed for counting blood cells, hence the name haemocytometer, it can also be used for counting cells/microorganisms in a liquid medium. It not only allows the counting of cells in suspension, but the percentage of viable (intact) cells can be determined using the dye exclusion method. Dead cells take up the dye trypan blue and appear blue under the microscope. Living cells exclude trypan blue, and appear white. Thus, the percentage of viable cells can be calculated.

Confluent HCAEC, HUVEC and HmCASM<sub>c</sub> cultures were washed with PBS, trypsinised and then centrifuged at 700g for 5 minutes to form cell pellets. The cell pellets were then resuspended in 200µl of growth medium to disaggregate the cells. Of this cell suspension 50µl was taken and mixed with 50µl trypan blue solution (0.4% w/v trypan blue in PBS) such that approximately 50-100 cells were observed over each of the 9 large squares of the haemocytometer chamber. The large squares in the haemocytometer have an area of 1 mm<sup>2</sup>. When the coverslip is passed over the grid, the depth of the chamber is 0.1 mm. Thus, the total volume over each large square = 1 x 1 x 0.1 = 0.1 mm<sup>3</sup> = 1x10<sup>-4</sup>ml. Cells were counted from 5 large squares and the mean cell number (n) was calculated. Since the cell suspension was mixed with the same volume of trypan blue, i.e. diluted 2-fold: the number of cells per ml in the suspension =  $n \times 10^4 \times 2$

From the cell number obtained from the haemocytometer the cell viability was calculated:

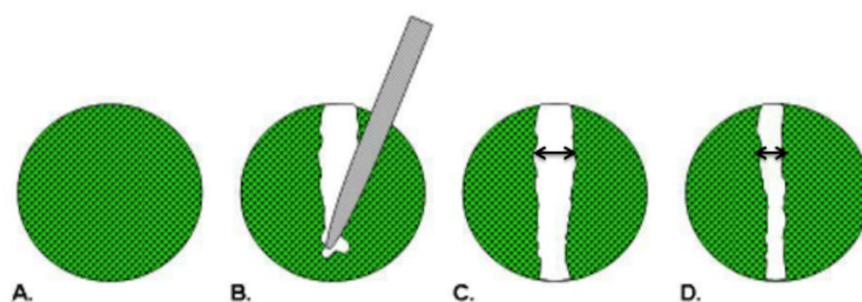
$$\% \text{Viability} = \frac{\text{Total number of viable cells}}{\text{Total number of viable and nonviable cells}} \times 100$$

### 2.2.6 Scratch-test assay

The scratch assay is a valuable tool for studying multi-cell migration in vitro and was conducted as a measure of cell growth (Liang et al., 2007). In this assay, which was first developed almost 50 years ago (Todaro et al., 1965), contact-inhibited cells are grown on a 2D surface until they form a confluent monolayer. A strip of cells is then removed by mechanically scraping the monolayer. Molecules of interest as potential therapeutics are added to the wells and images of cell movement are captured at regular intervals within a 24 hour period for data analysis (Yarrow et al., 2004; Yarrow et al., 2005). The monolayer responds with cell spreading and migration into the denuded region until the wound is closed.

HCAECs and HCASMCs were plated in 6 well plates in EGM-2 MV and SmGM-2 growth media respectively and allowed to grow to approximately 90% confluence. EGM-2 complete culture media was then removed and replaced with starvation media for 8 hours. A uniform scratch in the monolayer was then produced using a sterile pipette tip approximately 500 µm in width. Starvation media was replaced with 2ml of either complete growth media (EGM-2 MV for

HCAEC/SmGM-2 for HCASMC) or growth media containing 1 or 3 $\mu$ M nitrite and incubated in 1% hypoxia for 24 hours. Microscopic images were taken at 10x magnification after the scratch was made and then after 24 hours of treatment. Images were analyzed by determining the distance between cells on either side of scratch measured at baseline and at 24 hours. The absolute difference in scratch closure was then calculated for each treatment comparing 24 hours to baseline (Figure 2.21).



**Figure 2.21. Scratch Assay.**

A scratch was introduced into a confluent monolayer of cells (A) by drawing a tip across the cell layer (B). The denuded area is imaged to measure the boundary of the wound at pre-migration (C) and after cells have migrated inward to fill the gap (D).

### **2.2.7 Identification of potential nitrite reductases involved in nitrite-induced effects of cell growth/survival**

To investigate the role of specific nitrite reductases in 5% and 1% hypoxic conditions, HCASMCs, HCAECs and HUVECS were all incubated with the selective XOR inhibitor allopurinol (100 $\mu$ M) (Millar et al., 1998) both in the absence and presence of 3 $\mu$ M nitrite. To investigate the role of eNOS, HCAECs and HUVECS were treated with the NOS inhibitor L-nitro monomethyl arginine (L-NMMA; 300 $\mu$ M). All drug pretreatments were for 30 min prior to nitrite incubation. Additionally to test whether the effects of nitrite were cGMP mediated the selective soluble guanylate cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 1 mM) (Garthwaite et al., 1995) was added to a selection of 1% hypoxia experiments for both HCAECs and HCASMCs.

### **2.2.8 Determination of the expression of potential nitrite reductases and markers of cell growth**

To determine whether any of the treatments alter the expression of key nitrite reductases western blotting was conducted. In particular this technique was utilized to assess levels of eNOS, phosphorylated-eNOS (p-eNOS) and XOR in endothelial cells (HCAEC, HUVEC) and XOR in HmCASMCs. Additionally cell cycle markers (p21 and cyclin D1) were measured to see if the treatments altered their expression. This technique comprises 3 main steps; sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), gel transfer and incubation and detection of proteins with the appropriate antibodies.

#### **2.2.8.1 Sample Preparation for western blotting**

After treatments, HCAEC, HUVEC and HmCASM<sub>c</sub> cultures subjected to various treatments were washed with PBS, trypsinised (0.25%) and centrifuged at 700 g for 5 min. The supernatant was removed and the cell pellets snap frozen in liquid nitrogen and stored at -20°C until analysis. Following defrosting cell pellets were lysed using a stock solution of tissue lysis buffer (10mM Tris-HCL, 50mM NaCl, 30mM NaPPi and 2mM EDTA) and 0.5 M NaF, 1 % Triton X-100, 0.2 M Na<sub>3</sub>VO<sub>4</sub> as well as 1 µg/ml each of the protease inhibitors benzamidine, aprotinin, antipain, leupeptin, pepstatin A and AEBSF (Table 2.3).

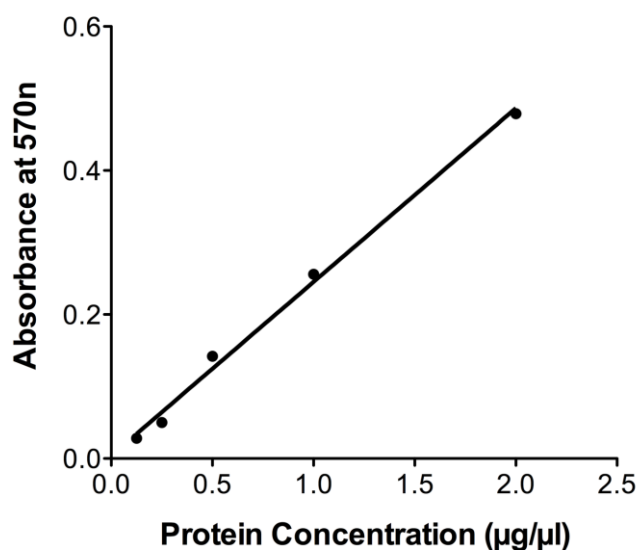


#### **2.2.8.2 Protein determination**

To quantify the amount of protein in each sample a Bradford Assay (Bradford, 1976) was performed. A standard curve was generated from bovine serum albumin (BSA, 2mg/ml) by serial dilution at 0.125-2 mg/ml (figure 2.23). 10  $\mu$ L of each standard and 10  $\mu$ L of each sample was loaded in duplicate on a 96-well plate. 200  $\mu$ L of reagent (Bio-Rad, UK; diluted 1:5 in MQ H<sub>2</sub>O) were added to each of the wells. The light absorbance was measured using a spectrophotometric plate reader (MRX-TC Revelation, Dynex Technologies, UK) at wavelength 570 nm. The protein concentration was determined by comparing the light absorbance of the unknown samples to the standards. The protein concentration of each sample was then adjusted to 20  $\mu$ g/ $\mu$ l in tissue lysis buffer.

Inhibitor	Source	Mechanism of action	Final conc.
Benzamidine	Sigma #B6506	Potent inhibitor of thrombin and trypsin	1 mg/l (5.7 $\mu$ M)
Antipain	Sigma #A6191	Reversible inhibitor of serine and cysteine proteases. Inhibits papain and trypsin more specificity than leupeptin. Plasmin is inhibited only slightly.	1 mg/l (1.5 $\mu$ M)
Aprotinin	Sigma #A1153	Basic single-chain polypeptide that inhibits numerous serine proteases by binding to the active site of the enzyme, forming tight complexes. It inhibits above all plasmin, kallikrein, trypsin, chymotrypsin and urokinase, but not carboxypeptidase A and B, papain, pepsin, subtilisin, thrombin and factor X. Used in cell culture to prevent proteolytic damage to cells and to extend lifetime of cells.	1 mg/l (0.15 $\mu$ M)
Leupeptin	Sigma #L0649	Tripeptide aldehyde. Reversible competitive inhibitor of serine and cysteine proteases.	2 mg/l (4.2 $\mu$ M)
Pepsatin A	Sigma #P5318	Pentapeptide derivative. Reversible inhibitor of aspartic proteases, e.g. pepsin, cathepsin D, chymosin, renin	0.7 mg/l (1.5 $\mu$ M)
AEBSF	Sigma #76307	Irreversible inhibitor of Thrombin and other serine proteases. Inhibits by acylation of the active site of the enzyme. Much less toxic than PMSF and DFP	100mg/l (400 $\mu$ M)

**Table 2.3 List of Protease inhibitors with their respective mechanism of action.**



**Figure 2.23 Typical standard curve generated from the Bradford protein assay.**

#### **2.2.8.3 SDS-PAGE**

Samples were diluted 1 in 5 in Laemmli's loading buffer (final concentration 0.05 M Tris-HCl, 6 % glycerol, 0.002 % bromophenol blue, 1.7 % SDS and 1.55 % DTT) and denatured by boiling at 95°C for 5 min. 10 % Mini-PROTEAN® TGX™ precast gels (Bio-Rad, Hertfordshire, UK) were used. The precast gel was securely locked in to a casting stand. From the gel, the comb was removed and the casting stand was placed inside a Mini-PROTEAN® TGX™ Tetra System gel tank. Running buffer (25 mM Tris-Base, 192 mM glycine, 0.1 % SDS) was then added to the gel tank until all wells were fully submerged and no air bubbles were present inside any well. 10 µl of protein marker (Precision Plus Protein™ Dual Colour Standards, 10 kDa – 250 kDa, Bio-Rad, Hertfordshire, UK) was loaded in to the first well followed by 30 µl of each diluted sample into subsequent wells providing an equal protein loading of each well. The proteins in the samples were then separated at 200 V for 90 min, i.e. until the dye was visible at the bottom of the gel.

#### **2.2.8.4 Protein transfer**

A piece of blot paper (PROTEAN®XL Size extra thick, Bio-Rad, Hertforshire, UK) and a Hybond™ Enhanced Chemiluminescence (ECL)™ nitrocellulose membrane (Amersham Biosciences, UK) was soaked in positive buffer (100 ml methanol + 18.3 g Tris-base, made up to 500 ml with water) for 20 min. A further piece of blot paper was soaked in negative buffer (100 ml methanol + 1.5 g Tris-base + 2.6 g 6-amino-n-caprioic acid, made up to 500 ml with water) for 20 min. The

positive blot paper was placed on a platinum anode followed by the nitrocellulose membrane, then the gel and finally the negative blot paper. In order to ensure homogenous transfer the 'stack' was rolled to ensure removal of air bubbles. A cathode was then placed on to the 'stack' and the proteins were then transferred from the gel to the membrane by electrotransfer at 100 mA using a Trans-Blot® SD semi-dry transfer cell (Bio-Rad, Herforshire, UK) for 75 min. Following this the membrane was stained with 0.1 % Ponceau S Solution. Ponceau S reversibly stains proteins so that the membrane can be visually inspected to ensure equal loading and transfer. With distilled water the Ponceau S was washed 3 - 5 times with gentle agitation on a Luckham R100 Rotatest Shaker (Ecomat, Reading, UK) for 3 min per wash step.

#### **2.2.8.5 Identification of proteins**

To determine the expression of proteins of interest the nitrocellulose membranes were then exposed to specific antibodies. Firstly, to prevent non-specific binding, each membrane was blocked for 1 hour at room temperature with gentle agitation with 5 % non-fat milk (Marvel®, Dublin, Republic of Ireland) in Tween-20 buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.6 and 0.05 % (w/v) Tween-20) and then washed with Tween-20 buffer. The membranes were then incubated overnight at 4°C with the primary antibody of interest (Table 2.4) diluted in 0.05 % (w/v) Tween-20 buffer. The following day, the membranes were washed again with Tween-20 buffer and incubated with the secondary antibody for an hour (Table 2.6).

### 2.2.8.6 Detection and quantification of proteins

In order to visualise the antibody-bound proteins on the membrane the Clarity™ Western ECL kit was used (Bio-Rad, Hertfordshire, UK). Solution A (peroxide solution) and solution B (Luminol / enhancer solution) were mixed in a 1:1 ratio and the membrane incubated in the mixture for 5 min. The excess was then drained and bands were visualized using a FluorChem E digital imager (proteinsimple, Santa Clara, CA, USA).

<u>Antibody</u>	<u>Concentration</u>
eNOS	1 <sup>o</sup> Ab: 1:2000 Rabbit Anti-human (Santa Cruz, Germany) 2 <sup>o</sup> Ab: 1:5000 Goat Anti-rabbit (Abcam, UK)
p-eNOS	1 <sup>o</sup> Ab: 1:1000 Rabbit Anti-human (Cell signalling, UK) 2 <sup>o</sup> Ab: 1:2000 Goat Anti-rabbit (Abcam, UK)
Anti-XOR	1 <sup>o</sup> Ab: 1:2000 Rabbit Anti-human (Abcam, UK) 2 <sup>o</sup> Ab: 1:3000 Goat Anti-rabbit (Abcam, UK)
p21	1 <sup>o</sup> Ab: 1:1000 Mouse Anti-human (BD Bioscience, UK ) 2 <sup>o</sup> Ab: 1:2000 Goat Anti-mouse (Abcam, UK)
Cyclin-D1	1 <sup>o</sup> Ab: 1:1000 Rabbit Anti-human (Cell signalling, UK) 2 <sup>o</sup> Ab: 1:2000 Goat Anti-rabbit (Abcam, UK)
Anti-β-actin	1 <sup>o</sup> Ab: 1:2000 Mouse monoclonal (Millipore, UK) 2 <sup>o</sup> Ab: 1:2000 Goat anti-mouse (Abcam, UK)

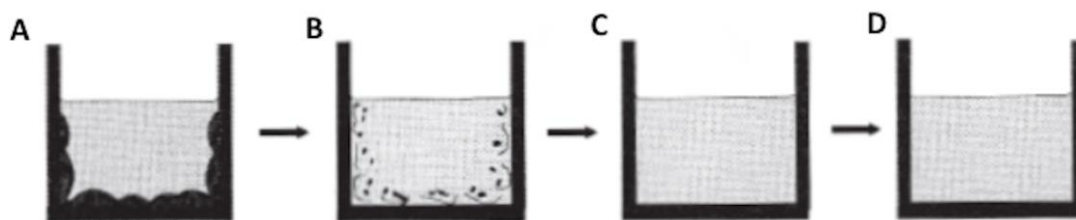
**Table 2.4. Primary and secondary antibodies used for western blot analysis.**

XO-Xanthine Oxidoreductase, e-NOS-endothelial nitric oxide synthase, P-eNOS- Phosphorylated endothelial nitric oxide synthase

Image J (National Institute of Health) was used to quantify the integrated density of protein on Western blots. For each blot the background signal was subtracted from experimental integrated densities to obtain sample values. Background subtracted values for nitrite reductase (eNOS, p-ENOS, XOR) or cell cycle (p21, cyclin D1) signal were then divided by background subtracted actin signal to obtain the HA/actin ratio.

### **2.2.9 Measurement of cGMP**

Levels of cGMP in cell samples was determined using an enzyme immunoassay (cGMP Enzymeimmunoassay Biotrak System RPN226, GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions ([www.gelifesciences.com](http://www.gelifesciences.com)), using a 96 well plate optical density reader. This process has been described previously in section 2.1.17 for the analysis of plasma cGMP. The main difference in the steps for the analysis, was the preparation of the pellets prior to analysis. For the cell pellets the samples were 1<sup>st</sup> lysed to enable analysis. Lysis reagent 1 (Dodecyltrimethylammonium bromide) was used to hydrolyse cell membranes releasing intracellular cGMP with the 2<sup>nd</sup> lysis reagent sequestering the key component in lysis reagent 1 and ensuring cGMP is free for subsequent analysis. Lysis reagent 1 was added to cultured cells followed by a 5 minute incubation before assay (fig 2.20). The antiserum is reconstituted with lysis reagent 2 rather than assay buffer as performed in section 2.1.17. Samples measured were then compared to a standard curve generated from known standards.



**Figure 2.22. Cell lysis protocol**

- A. Cultured cells present in 6 well plates were decanted and washed
- B. Cells were lysed using lysis reagent 1.
- C. Cells were transferred to new plate
- D. Assay was performed

### 2.2.10 Data and statistical analyses

All data were analysed using GraphPad Prism version 5.0 for Mac OsX (GraphPad Software, San Diego, CA; [www.graphpad.com](http://www.graphpad.com)). All p values are 2 sided and significance accepted as  $p < 0.05$ . All values are expressed as mean  $\pm$  standard error of the mean (SEM) with the  $n$  value representing the experiments in that particular treatment group. For cell number, viability, cGMP levels and expression of nitrite reductases/cell cycle markers one-way ANOVA with Bonferroni post-hoc tests for individual group comparisons was used. Determinations of correlations were performed using the Pearson's correlation coefficient analysis of least-squares.

# **Chapter 3.0**

## **Investigation of the safety and efficacy of intracoronary nitrite infusion during primary PCI for acute myocardial infarction**



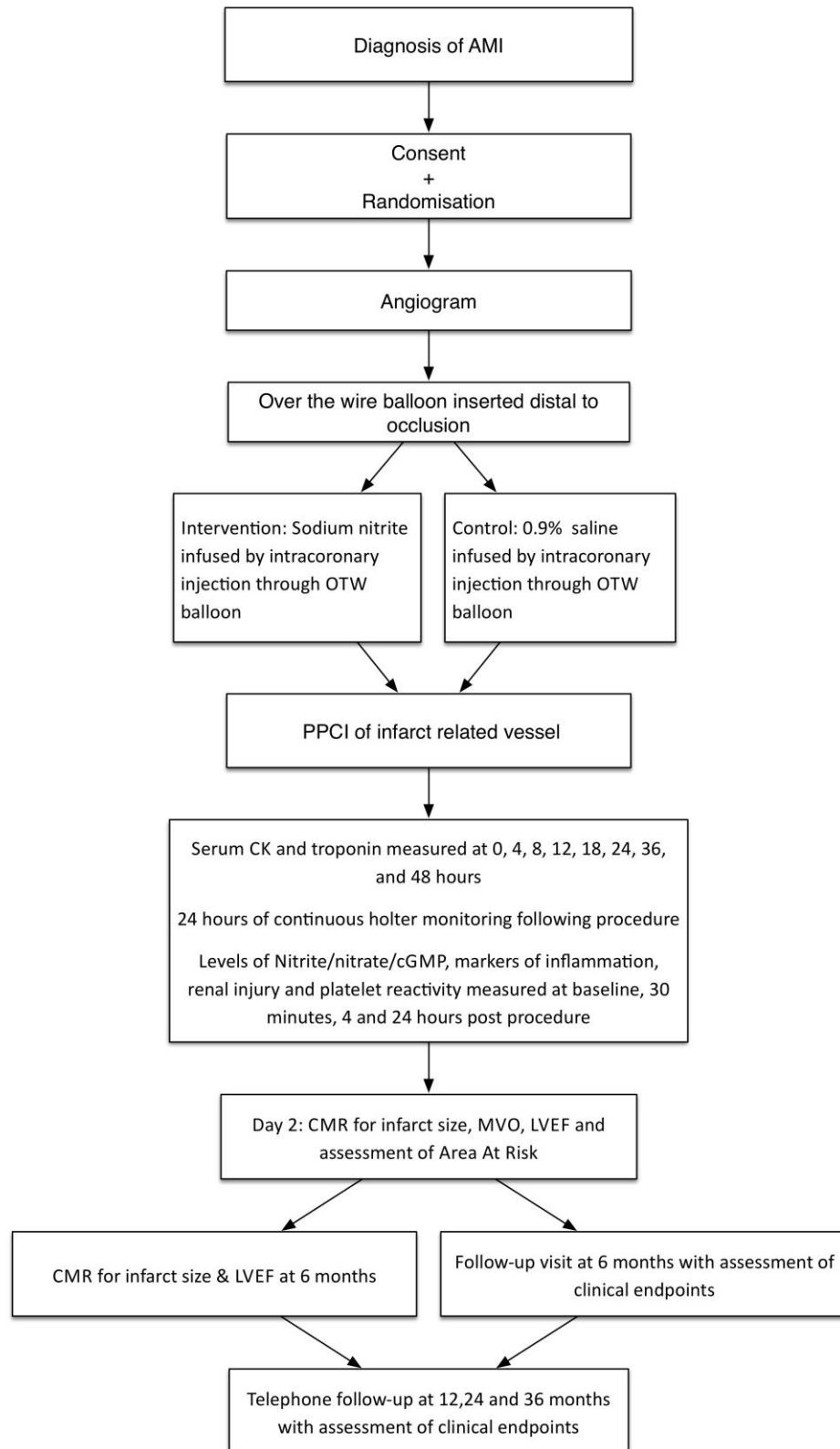
### 3.1 Introduction

Reperfusion injury remains a limitation to the beneficial effects of primary PCI for STEMI (Frolich et al 2013). A potential solution has emerged in the form of inorganic nitrite. The activity of nitrite resides in its propensity for conversion to NO under the optimal conditions of low pO<sub>2</sub> and low pH (Gladwin et al 2005), conditions that prevail during ischaemic episodes. NO exerts a number of actions (Moncada & Higgs 1993) that are thought to underlie its cardioprotective effects (Johnson et al 1991) including anti-inflammatory and anti-platelet actions (Jones & Bolli 2006) and the prevention of the opening of the mitochondrial permeability transition pore (Heusch et al 2008). Pre-clinical evidence has shown that nitrite administered either intra-ventricular, intra-coronary but also intravenously reduces infarct size (Webb et al 2004; Baker et al 2007; Gonzalez et al 2008). In these studies the beneficial effects of nitrite were shown to be due to the activity of NO and were often associated with local application of nitrite, prior to reperfusion, into or on the organ of interest. These observations support investigation of the potential of nitrite in the treatment of acute STEMI, particularly where it could be delivered locally before balloon inflation at the time of primary PCI.

Thus the aim of the study described in this chapter is to assess whether intra-coronary nitrite is safe, tolerable and reduces infarct size during primary PCI in patients with STEMI as per the study design described in chapter 2, section 1 and Figure 3.1.

### **3.2 Power analysis**

It was hypothesised that nitrite would reduce the AUC for creatine kinase release by 30%, the same amount as both cyclosporine (Piot et al., 2008) and postconditioning (Staat et al., 2005). For a statistical power of 80% and a probability of a type I error of 0.05 using a two-sided test, it was calculated that the sample size should be 70 subjects (35 per group). It was further estimated that 4-8% of patients would die by the time of the endpoint at 6 months and 10% would either not tolerate or fail to attend the CMR scan at 6 months so an additional 10 patients were recruited to account for these eventualities, giving a total of sample of 80 patients.



**Figure 3.1. Flow diagram of Study design**

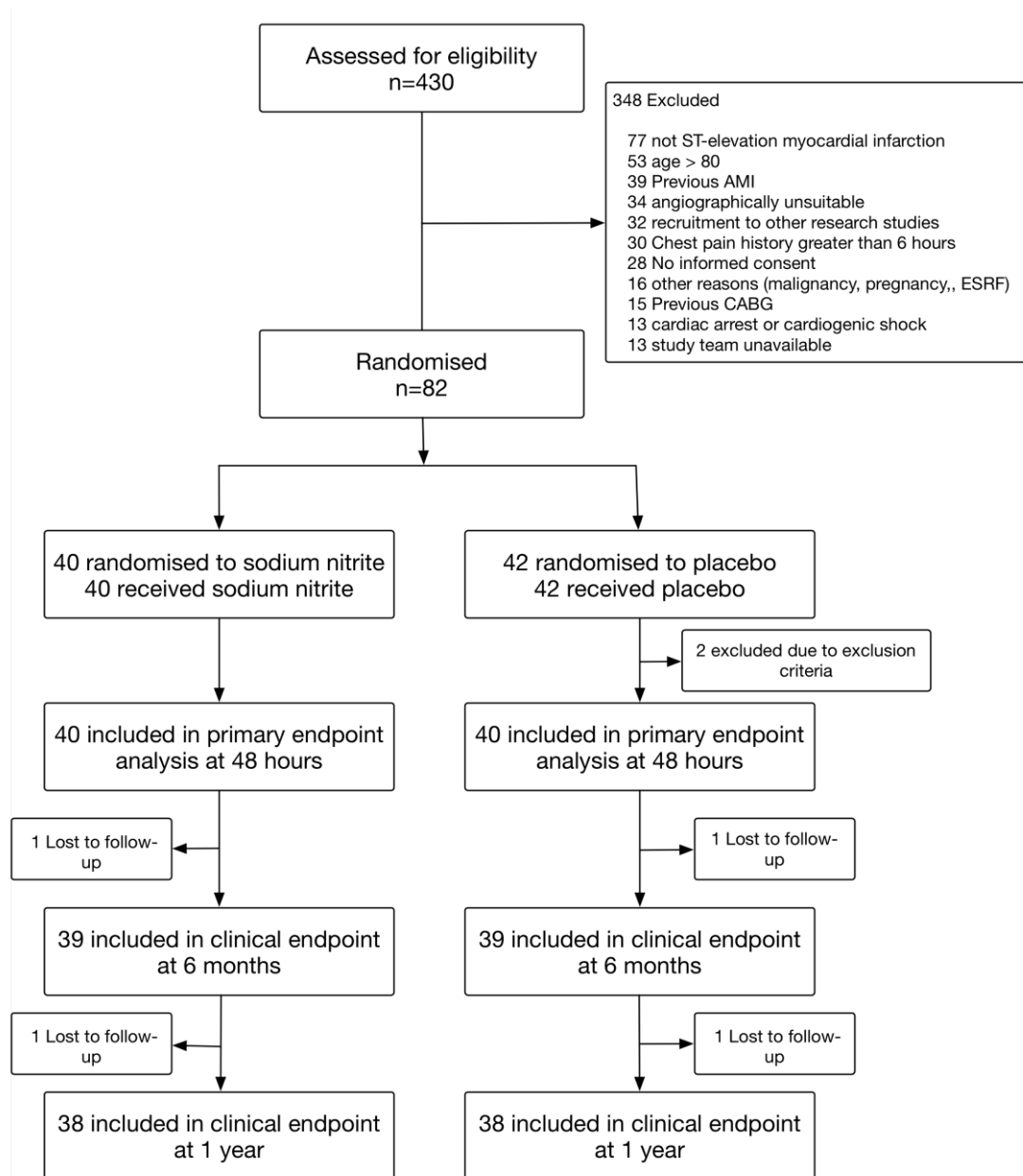
AMI: acute myocardial infarction, OTW: Over the wire balloon, PPCI: primary percutaneous coronary intervention, CMR: cardiac magnetic resonance imaging, MVO: microvascular obstruction, LVEF: Left ventricular ejection fraction

### **3.3 Results**

#### **3.3.1 General characteristics of Study population**

Between April 2012 and December 2012, 430 patients were hospitalised for management of AMI at The Barts Health Heart Attack Centre. Of these patients 353 underwent PCI. Among these 353 patients, 13 were not evaluated for enrollment because study personnel were not available. Another 251 were evaluated and excluded for the following reasons: onset of chest pain more than 6 hours before presentation (30), greater than 80 years of age (53), preadmission cardiac arrest or cardiogenic shock before primary PCI (13), previous AMI in the same territory (39), previous CABG (15), recruitment to other research studies (32), unable to consent (19), angiographically unsuitable (34), and other (malignancy, renal failure, pregnancy (16)). This left 89 suitable patients, of which 9 declined. Data are thus presented for 80 patients (40 in the control group and 40 in the nitrite group, Figure 3.2).

Patients randomised to nitrite infusion had a longer ischaemia time than those who received placebo ( $207.05 \pm 76.35$  minutes vs  $171.63 \pm 67.72$  minutes). All other baseline characteristics were similar between the treatment groups (Table 3.1). The mean age of the trial participants was 57 years, with 84% male. The two study groups were similar with respect to syntax score, culprit vessel, baseline TIMI flow and morphine use pre-PCI (Table 3.1).



**Figure 3.2 Consort diagram.**

	Nitrite (n=40)	Placebo (n=40)	P Value
Age (yr) (Mean±SD)	56.35±11.16	57.60±13.20	0.649
Sex (M/F)	36/4	31/9	0.225
Diabetes mellitus	3 (7.5%)	3 (7.5%)	1.000
Body-mass index (kg/m <sup>2</sup> ) (Mean±SD) <sup>a</sup>	28.97±5.14	28.58±5.17	0.740
Hypertension	20 (50.0%)	14 (35.0%)	0.258
Hypercholesterolaemia	16 (40.0%)	12 (30.0%)	0.482
Heart rate (BPM) (Mean±SD)	72.68±18.62	77.35±21.31	0.299
Systolic BP (mmHg) (Mean±SD)	124.48±29.92	136.13±26.99	0.071
Ischaemia time (min) (Mean±SD) <sup>b</sup>	207.05±76.34	171.63±67.72	0.031
Culprit Vessel			0.736
Left anterior descending	9 (22.5%)	12(30%)	
Circumflex	5 (12.5%)	5 (12.5%)	
Right coronary	26 (65.0%)	23 (57.5%)	
TIMI flow before PCI			0.451
0	30 (75.0%)	31 (77.5%)	
1	6 (15.0%)	3 (7.5%)	
2	2 (5.0%)	5 (12.5%)	
3	2 (5.0%)	1 (2.5%)	
0/1	36 (90.0%)	34 (85.0%)	
Treatment before PCI			
Morphine	26 (43.3%)	34 (56.7%)	0.070

**Table 3.1 Baseline characteristics by treatment group.**

Values shown as number (%) unless otherwise stated. (PCI, percutaneous coronary intervention; TIMI, Thrombolysis in myocardial infarction; DES drug-eluting stent)

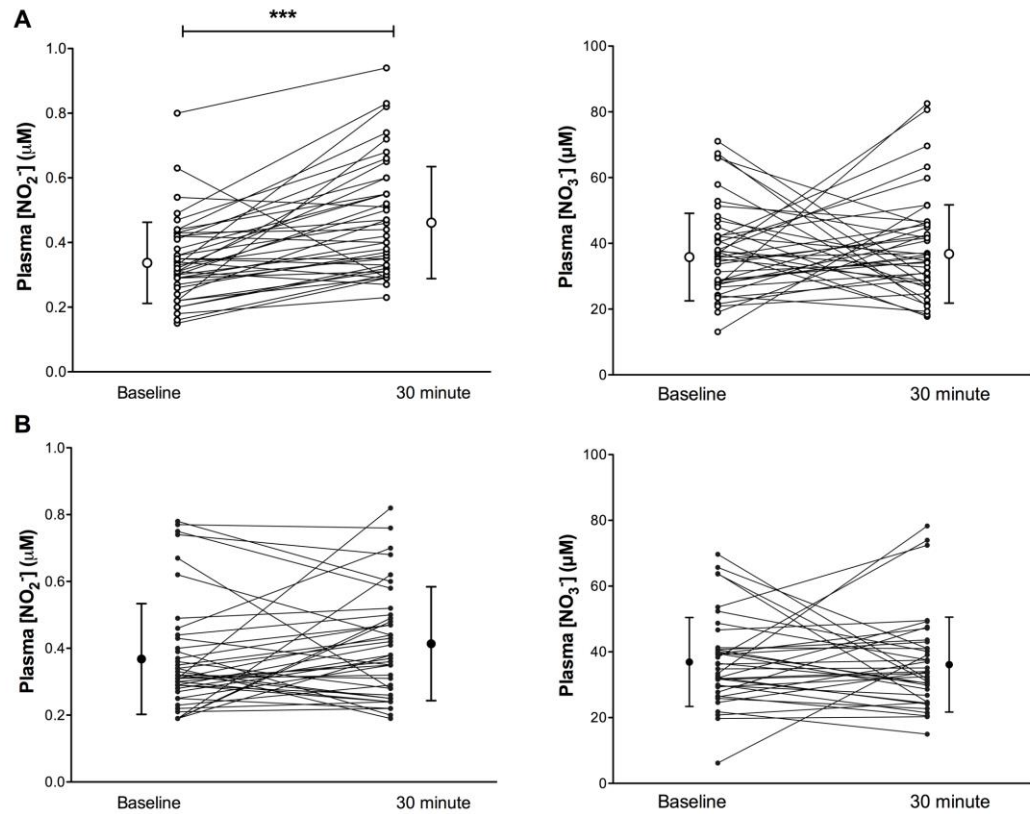
<sup>a</sup> The body-mass index is the weight in kilograms divided by the square of the height in meters.

<sup>b</sup> Ischaemia time determined from symptom to balloon times for each patient.

Area at risk scores were non-significantly higher in the nitrite group (APPROACH: 30.88 (95%CI: 28.30 to 33.47) vs 26.63 (95%CI: 23.28 to 29.99); BARI: 27.41 (95% CI: 24.02 to 30.80) vs 25.17 (95%CI 22.20 to 28.13) (Table 3.2). Stenting of the culprit lesion was performed in 97.5% of all patients. Use of thrombectomy catheters, drug-eluting stents and anti-platelet therapy were similar between the 2 groups. Mean contrast loads were similar between the 2 groups (nitrite 261.2±15.61mls vs placebo 236.3±12.05mls, p=0.211). In five patients, TIMI 3 flow was not achieved after PCI (3 in the nitrite group and 2 in placebo), however levels of ST segment resolution (>70%) were the same in the 2 groups (88.5%).

### **3.3.2 Plasma NOx**

Measurement of nitrite levels locally post-intervention during angioplasty is difficult. In addition evidence indicates that induction of iNOS during I/R may interfere with measurement. However samples were collected for analysis at 30 minutes post infusion. Figure 3.3 demonstrates that whilst there were no differences in plasma nitrate or nitrite at baseline, 30 minutes following angioplasty, circulating NO<sub>2</sub><sup>-</sup> but not NO<sub>3</sub><sup>-</sup> levels did rise indicating successful nitrite administration.



**Figure 3.3 Plasma  $\text{NO}_2^-$  and  $\text{NO}_3^-$  levels pre and post study infusion**

Dot plots showing plasma  $\text{NO}_2^-$  and  $\text{NO}_3^-$  levels measured at baseline and 30 minutes after delivery of either intra-coronary nitrite or placebo in all patients. Each line representing the difference between baseline and 30 minute plasma  $\text{NO}_2^-$  and  $\text{NO}_3^-$  levels is shown for each patient in the nitrite group in panel A and placebo in panel B. Error bars represent mean  $\pm$  SD for each group. \*\*\* $P < 0.0001$  using paired T-test. ( $\text{NO}_2^-$  = Nitrite,  $\text{NO}_3^-$  = Nitrate)



	Nitrite (n=40)	Placebo (n=40)	P value
Access site			
Radial	31 (77.5%)	28 (70%)	0.612
Syntax Score	13.41±5.50	13.58±6.20	0.893
DES use	33 (82.5%)	30 (78.9%)	0.778
Thrombectomy use	33 (82.5%)	31 (77.5%)	0.781
Contrast use (mls)	261.20±15.61	236.30±12.05	0.211
Angiographic AAR			
APPROACH (Mean (95% CI))	30.89 (28.30-33.47)	26.63 (23.28-29.99)	0.050
BARI (Mean (95% CI))	27.41 (24.02-30.80)	25.17 (22.20-28.13)	0.316
Treatment at time of PCI			
Heparin	40 (100%)	40 (100%)	1.000
Aspirin	40 (100%)	40 (100%)	1.000
Clopidogrel/Prasugrel	35/5	37/3	0.712
GPIIb/IIIa inhibitor	40 (100%)	40 (100%)	1.000
GTN use	18 (45%)	18 (45%)	1.000
ST segment resolution>70%	35 (88.5%)	35 (88.5%)	1.000
Procedural Success	37 (92.5%)	38 (95.0%)	0.615

**Table 3.2. Procedural characteristics by treatment group.**

Values shown as number (%) unless otherwise stated. (AAR, area at risk; DES, Drug-eluting stent; GTN, Glyceryl Trinitrate; GP, glycoprotein)

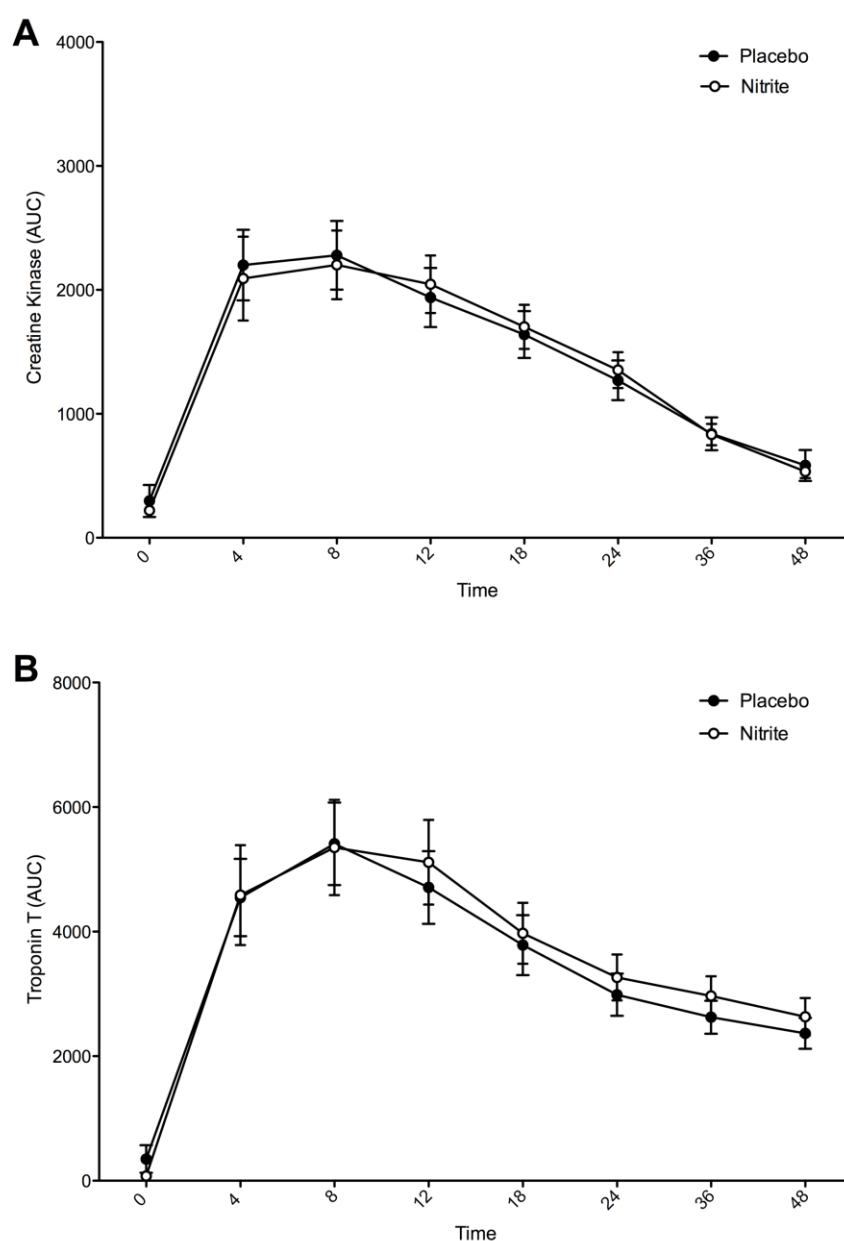
### 3.3.3 Infarct size

There was no evidence of difference in the AUC for serum CK release after reperfusion between the nitrite and control groups, with a median of 56,398 arbitrary units (IQR: 31185 to 83,531) in the nitrite group versus 48,195 (IQR: 27,726 to 82,841) in the control group ( $p=0.92$ ) (Figure 3.4A). The median AUC for troponin T release was 140,782 arbitrary units (IQR: 84,949 to 218,133) in the nitrite group and 136,412 arbitrary units (IQR: 70,045 to 239,483) in the control group. This difference was also not conventionally statistically significant ( $p = 0.85$ ) (Figure 3.4B). Ischaemia time correlated significantly with infarct size (CK:  $r=0.256$  and  $p=0.022$ , Troponin T:  $r=0.273$  and  $p=0.014$ , Figure 3.5)

### 3.3.4 CMR analysis

Of the 80 patients recruited 12 declined consent for the CMR protocols. In the remaining 68 patients (33 in nitrite group and 35 in placebo) no evidence of difference in left ventricular volumes (end-diastolic volume, end-systolic volume), left ventricular mass or ejection fraction (LVEF) between the nitrite and placebo treated groups were evident (Table 3.3). There was a trend to smaller infarct size, incidence and amount of microvascular obstruction in the nitrite-treated group compared to placebo. CMR assessed infarct size was positively correlated with cardiac biomarkers (CK,  $r=0.770$ ,  $p<0.01$ ; troponin T,  $r=0.787$ ,  $p<0.01$ , Figure 3.6). There was a trend to a higher area at risk in the nitrite group compared to placebo (Figure 3.7) but this was not significant. There was also a

trend to a higher MSI but again this difference was on the borders of statistical significance ( $p=0.05$ ).



**Figure 3.4. Assessment of Infarct size by biomarker assessment**

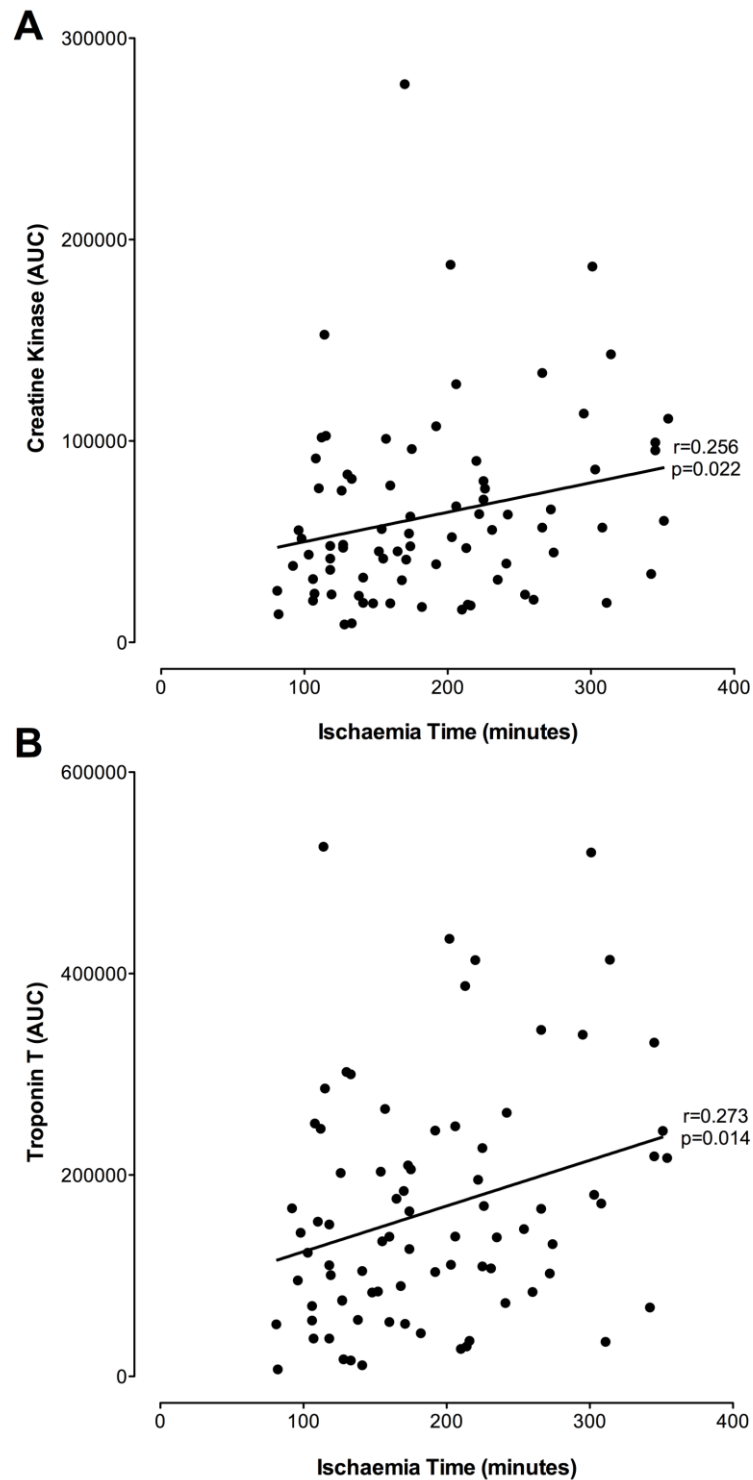
Serum CK was measured at baseline, 4, 8, 12, 18, 24, 36 and 48 hours after coronary reperfusion. Curves for the nitrite and control group are shown in Panel A. There was no difference in infarct size as measured by CK release between the 2 groups. Serum troponin T was measured at the same time points as creatine kinase. Curves for the nitrite ( $n=40$ ) and control ( $n=40$ ) groups are shown in Panel B. Nitrite administration did not result in a significant reduction in infarct size as measured by troponin T release. T bars denote standard errors of the mean (SEM) (CK = Creatine Kinase).

The CMR assessed area at risk correlated significantly with both angiographic risk scores (APPROACH:  $r=0.678$  and  $p<0.01$ , BARI:  $r=0.541$  and  $p<0.01$ , Figure 3.8).

	Nitrite	Placebo	P value
<b>Baseline CMR</b>	<b>(n=33)</b>	<b>(n=35)</b>	
LVEDVi	76.13 (71.11-81.14)	70.58 (65.22-75.94)	0.13
LVESVi	36.16 (32.56-39.75)	35.71 (30.94-40.48)	0.88
LVMi	63.02 (58.65-67.40)	58.23 (54.67-61.79)	0.09
LVEF	52.87 (49.88-55.86)	50.07 (46.46-53.67)	0.23
Infarct size (% LV)	17.10 (14.12-20.08)	19.55 (16.40-22.70)	0.25
AAR	34.58 (31.62-37.55)	33.05 (29.40-36.72)	0.52
MSI	0.52 (0.46-0.58)	0.44 (0.39-0.49)	0.05
MVO			
No. (%)	16 (48.5%)	23 (69.7%)	0.13
Amount (median (IQR))	2.98 (0-6.25)	3.47 (0-4.75)	0.34
<b>6 month CMR</b>	<b>(n=29)</b>	<b>(n=33)</b>	
LVEDVi	82.13 (74.80-89.46)	75.62 (69.90-81.34)	0.15
LVESVi	36.50 (31.48-41.52)	34.85 (30.53-39.17)	0.61
LVMi	55.67 (51.66-59.68)	51.20 (48.17-54.24)	0.07
LVEF	55.93 (52.71-59.15)	54.75 (51.62-57.87)	0.59
Infarct size (% LV)	11.88 (9.52-14.24)	13.15 (10.75-15.56)	0.45

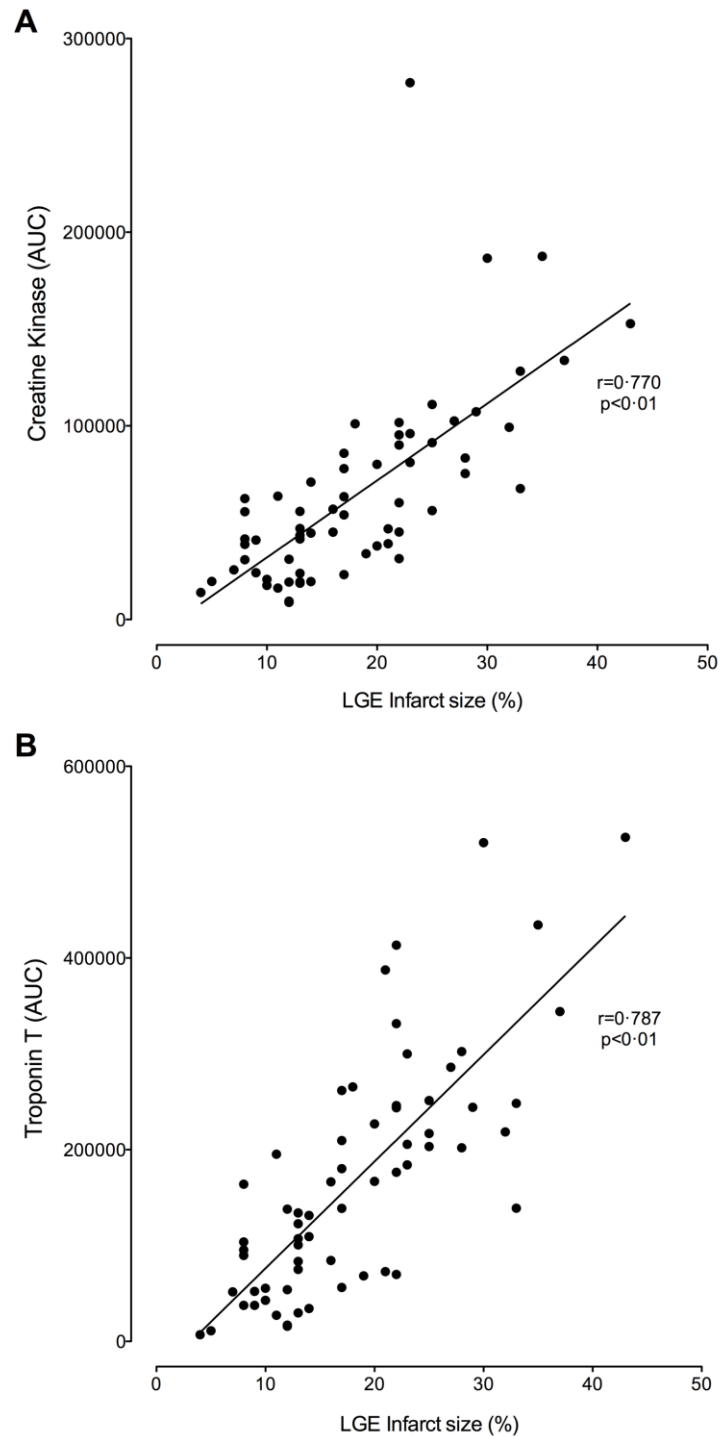
**Table 3.3 Cardiac magnetic resonance data for study population**

Values shown as Mean (95% CI) unless otherwise stated. (LVEDVi, Indexed left ventricle end-diastolic volume; LVESVi, Indexed left ventricle end-systolic volume; LVMi, Indexed left ventricle mass; LVEF, left ventricle ejection fraction; AAR, area at risk; MSI, myocardial salvage index; MVO, microvascular obstruction)



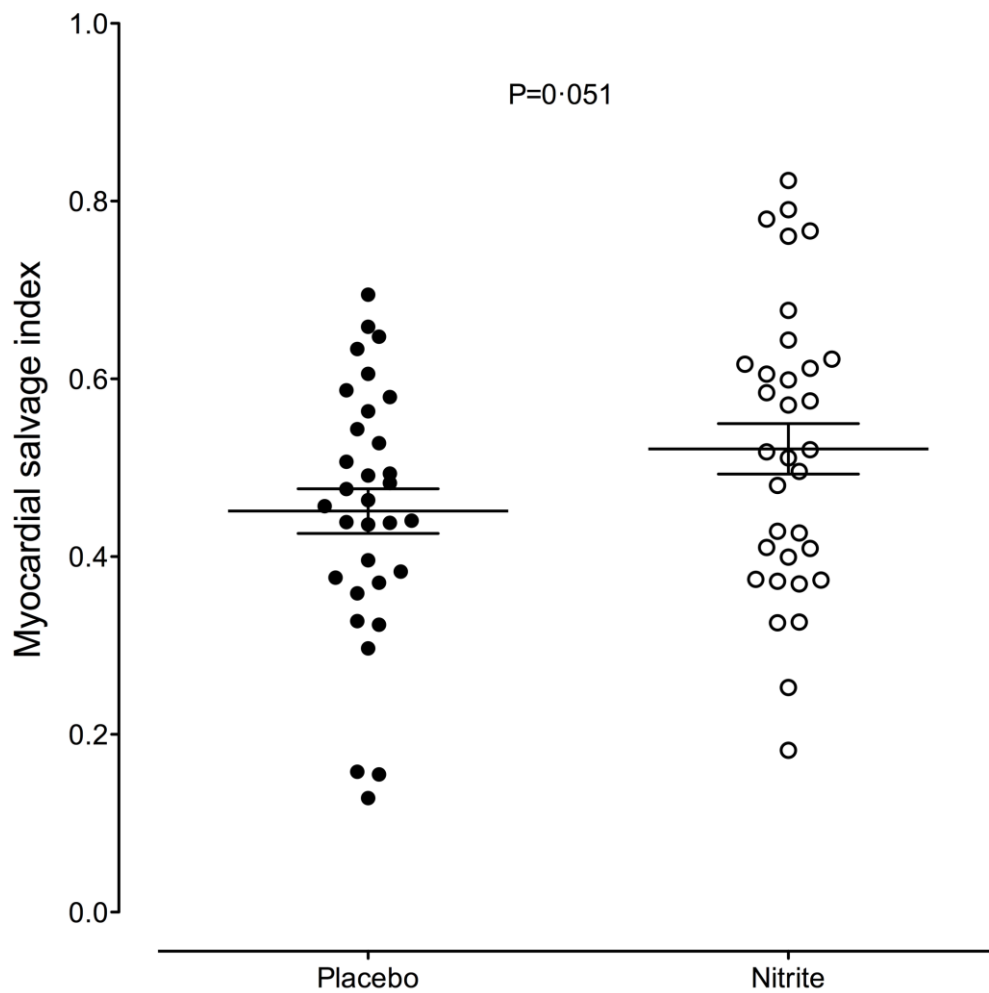
**Figure 3.5 Associations between cardiac biomarker assessment of infarct size and ischaemia time**

There was a significant positive correlation between infarct size assessed by creatine kinase area under the curve (AUC) and ischaemia time ( $r=0.256$ ), as shown in panel A. Panel B depicts a similar positive association between troponin T AUC and ischaemia time ( $r=0.273$ ). Associations determined using Pearson's correlation coefficient assessment.



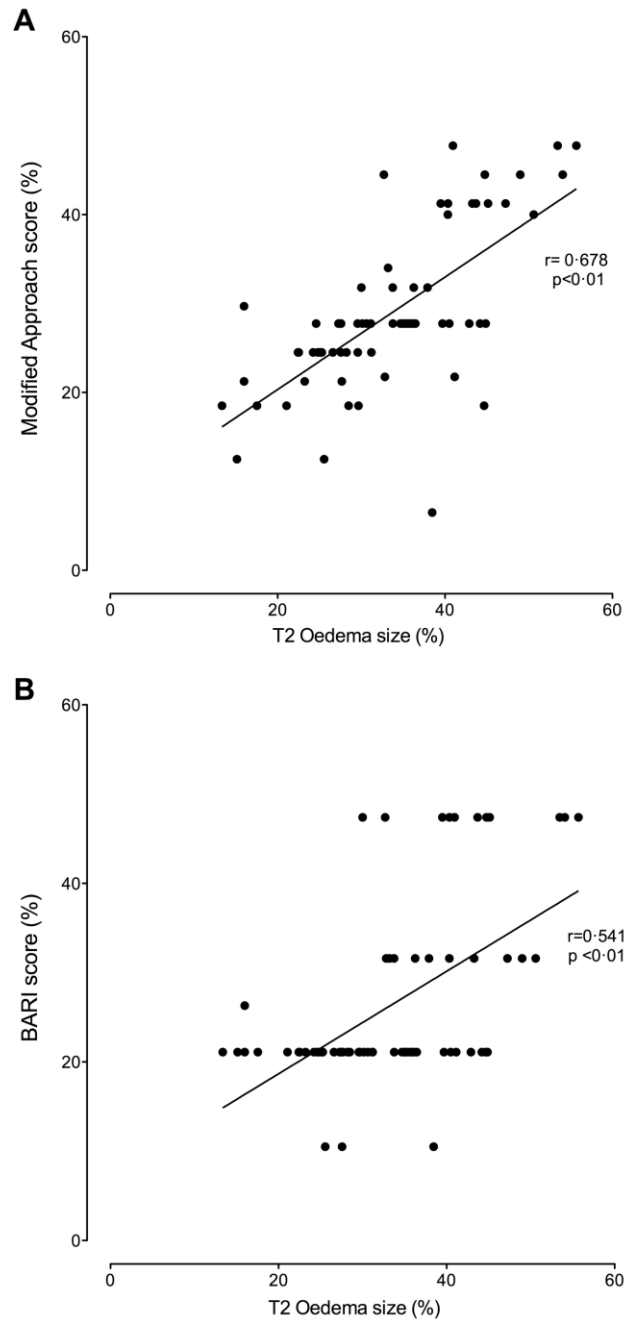
**Figure 3.6 Associations between cardiac biomarker assessment of infarct size and infarct size on cardiac magnetic resonance imaging**

There was a significant positive correlation between infarct size assessed by creatine kinase area under the curve (AUC) and LGE (late gadolinium enhancement) assessed infarct size on CMR (late gadolinium enhancement) ( $r=0.770$ ), as shown in panel A. Panel B depicts a similar positive association between troponin T AUC and LGE CMR infarct size ( $r=0.787$ ). Associations determined using Pearson's correlation coefficient assessment.



**Figure 3.7 Assessment of myocardial salvage index on CMR.**

The size of the myocardial salvage index on CMR is presented for 35 TIMI flow 0-3 patients in the control group and 33 patients in the nitrite group. The mean index as assessed by CMR was larger in the nitrite group than in the control group and is on the margins of conventional statistical significance. Significance evaluated using unpaired t test and data shown as mean  $\pm$  SEM.



**Figure 3.8 Associations between angiographic area at risk scores and area at risk assessed by cardiac magnetic resonance imaging (CMR)**

There was a significant positive correlation between the angiographic area at risk as assessed by the modified APPROACH score and the area at risk assessed by T2 oedema imaging on CMR ( $r=0.678$ ), as shown in panel A. Panel B depicts a similar positive association between the modified BARI score and the area at risk assessed by T2 oedema imaging on CMR ( $r=0.541$ ). Associations determined using Pearson's correlation coefficient assessment.



### **3.3.5 Subgroup analysis (TIMI flow and successful procedures)**

We conducted a single sub-group analysis on the 66 patients with TIMI flow  $\leq 1$  pre-procedure and a successful procedure i.e. drug delivered. In this sub-group ischaemia time was the same between the two groups as were all other baseline characteristics (Table 3.4).

#### **3.3.5.1 Infarct size in subgroup**

Importantly, there was a significant reduction in myocardial infarct size assessed by CK AUC between the nitrite and control groups, with a median of 44,608 arbitrary units (IQR: 27,535 to 64,848) in the nitrite group versus 55,666 (IQR: 41,591 to 93,659) in the control group ( $p=0.030$ ). This represents a 19% reduction in infarct size (Figure 3.9A). The median AUC for troponin T release was 131,410 AU (IQR: 71,337 to 183,452) in the nitrite group and 176,492 AU (IQR: 89,831 to 245,094) in the control group. This difference is not conventionally statistically significant ( $p=0.158$ ) (Figure 3.9B).

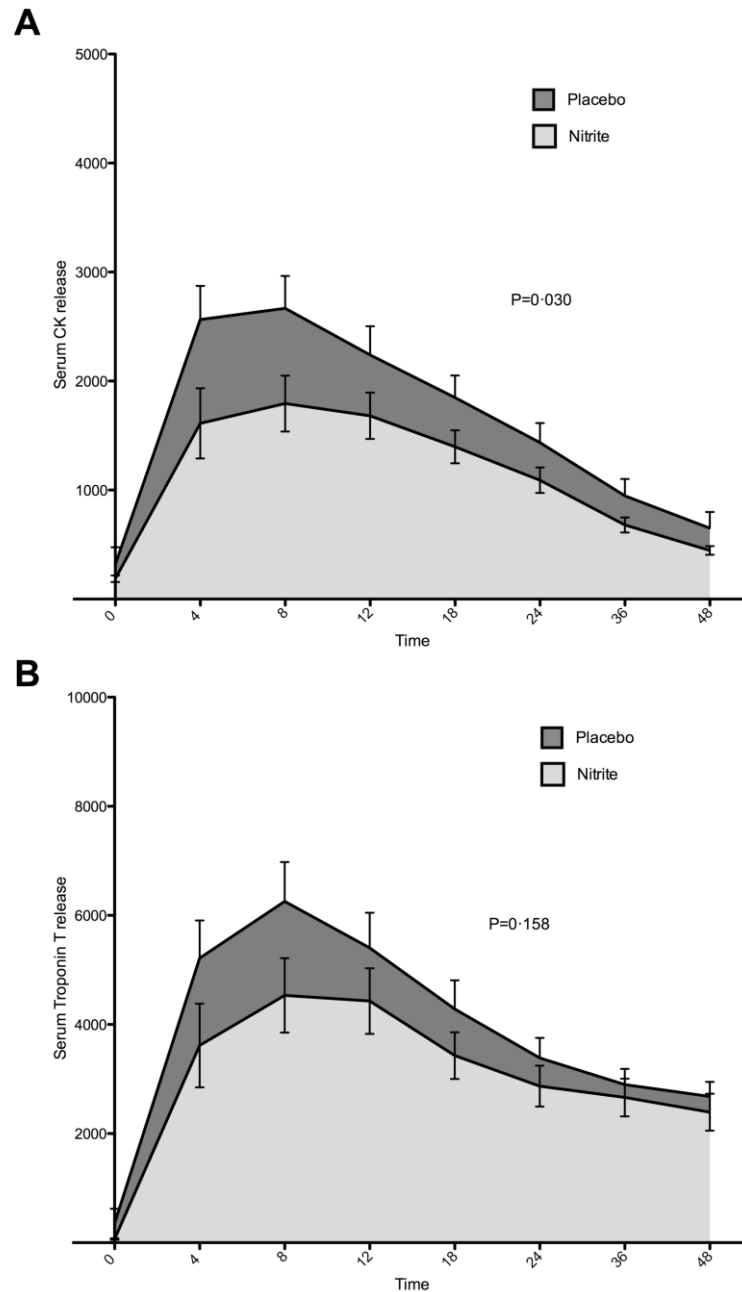
In the nine patients with TIMI  $>1$  flow at time of infusion, baseline characteristics were similar between the groups aside from a significantly longer ischaemia time in the nitrite group ( $286.80 \pm 96.70$  vs  $174.33 \pm 55.19$  minutes,  $p=0.038$ ) (Table 3.5). There was no statistical difference in infarct size assessed by CK (78398 IQR: 30945-104752 vs 21196 IQR: 13864-73887,  $p=0.413$ ) or troponin T AUC (144798 IQR: 59452-207770 vs 83796

IQR: 55473-182372) seen in the patients with TIMI flow >1 treated with nitrite compared to placebo, although there was a trend to increased values in the nitrite group.

	Nitrite (n=33)	Placebo (n = 33)	P value
Age (yrs) (mean±SD)	57.30±11.29	56.94±13.48	0.90
Sex (male/female)	29/4	28/5	0.99
Diabetes mellitus	2 (6.1)	1 (3.0)	0.99
Body-mass index (kg/m <sup>2</sup> ) (mean±SD) <sup>a</sup>	29.27±5.30	29.06±5.17	0.87
Hypertension	16 (48.5%)	8 (24.2%)	0.07
Hypercholesterolaemia	13 (39.4%)	10 (30.3%)	0.61
Heart rate (mean±SD)	70.94±18.97	79.06±22.10	0.11
Systolic Blood pressure (mean±SD)	120.76±29.64	132.94±23.44	0.07
Ischaemia time (minutes) (mean±SD) <sup>b</sup>	194.45±69.05	168.63±69.94	0.11
Culprit Vessel			0.57
Left anterior descending	8 (24.2%)	9 (27.3%)	
Circumflex	3 (9.1%)	5 (12.5%)	
Right coronary	22 (66.7%)	19 (57.6%)	
Syntax score (mean±SD)	13.29±5.42	13.68±5.42	0.77
DES use	29 (87.9%)	26 (81.3%)	0.51
Angiographic AAR			
APPROACH (mean±SD)	30.72±7.75	26.94±10.73	0.11
BARI (mean±SD)	27.32±10.88	24.75±9.26	0.31
Treatment before PCI			
Morphine	22 (66.7)	29 (87.9)	0.08
Treatment at time of PCI			
Heparin	33 (100)	33 (100)	0.99
Aspirin	33 (100)	33 (100)	0.99
Clopidogrel/Prasugrel (no/no)	29/4	30/3	0.99
Glycoprotein IIb/IIIa inhibitor	33 (100)	33 (100)	0.99

**Table 3.4. Baseline characteristics of the TIMI <1 subgroup**

Values shown as number (%) unless otherwise stated. Abbreviations: PCI, percutaneous coronary intervention; TIMI, Thrombolysis in myocardial infarction; DES, drug-eluting stent. <sup>a</sup> The body-mass index is the weight in kilograms divided by the square of the height in meters. <sup>b</sup> Ischaemia time determined from symptom to balloon times for each patient.



**Figure 3.9 Assessment of Infarct size by biomarker assessment in the TIMI flow  $\leq 1$  subgroup**

Serum CK was measured at baseline, 4, 8, 12, 18, 24, 36 and 48 hours after coronary reperfusion. Curves for the nitrite and control group are shown in Panel A. Nitrite administration resulted in a significant reduction in infarct size of approximately 20% as measured by CK release. Serum troponin T was measured at the same time points as CK. Curves for the nitrite (n=33) and control (n=33) groups are shown in Panel B. Nitrite administration did not result in a significant reduction in infarct size as measured by troponin T release. T bars denote standard errors of the mean (SEM).

	Nitrite (n=4)	Placebo (n = 5)	P value
Age (yrs) (mean±SD)	54.75±5.50	60.00±13.87	0.50
Sex (male/female)	4/0	2/3	0.17
Diabetes mellitus	1 (25.0)	2 (40.0)	0.99
Body-mass index (kg/m <sup>2</sup> ) (mean±SD) <sup>a</sup>	28.53±5.43	25.95±5.85	0.54
Hypertension	3 (75.0%)	4 (80.0%)	0.99
Hypercholesterolaemia	3 (75.0%)	1 (25.0%)	0.21
Heart rate (mean±SD)	74.00±17.38	72.80±13.41	0.91
Systolic Blood pressure (mean±SD)	131.50±26.90	135.80±33.82	0.84
Ischaemia time (minutes) (mean±SD) <sup>b</sup>	286.80±96.70	174.33±55.19	0.04
Culprit Vessel			0.49
Left anterior descending	1 (25.0%)	2 (40.0%)	
Circumflex	1 (25.0%)	0 (0.0%)	
Right coronary	2 (50.0%)	3 (60.0%)	
Syntax score (mean±SD)	15.63±7.91	13.30±10.79	0.73
DES use	1 (25.0%)	2 (40.0%)	0.99
Angiographic AAR			
APPROACH (mean±SD)	31.68±10.15	25.18±9.98	0.30
BARI (mean±SD)	26.35±6.06	24.24±4.69	0.57
Treatment before PCI			
Morphine	3 (75.0)	4 (80.0)	0.99
Treatment at time of PCI			
Heparin	4 (100)	5 (100)	0.99
Aspirin	4 (100)	5 (100)	0.99
Clopidogrel/Prasugrel (No/No)	3/1	5/0	0.44
Glycoprotein IIb/IIIa inhibitor (%)	4 (100)	5 (100)	0.99

**Table 3.5 Characteristics and infarct size of the TIMI >1 subgroup.**

Values shown as number (%) unless otherwise stated. Abbreviations: PCI, percutaneous coronary intervention; TIMI, Thrombolysis in myocardial infarction; DES, drug-eluting stent. <sup>a</sup> The body-mass index is the weight in kilograms divided by the square of the height in meters. <sup>b</sup> Ischaemia time determined from symptom to balloon times for each patient.

### 3.3.5.2 CMR analyses in subgroup

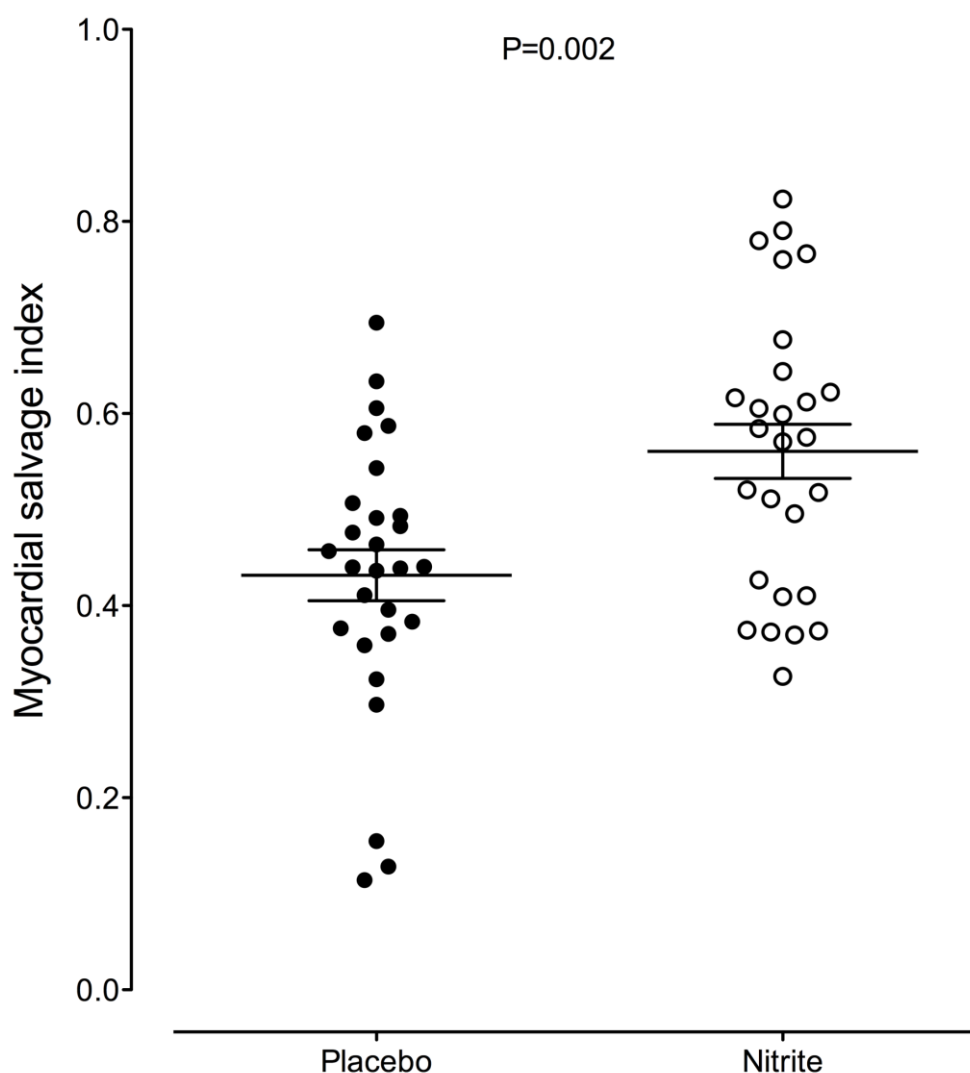
In the TIMI flow  $\leq 1$  group for the CMR analysis nitrite treatment was associated with a 25% decrease in myocardial infarct size (15.31 vs 20.08,  $p=0.03$ ) and a 30% increase in the myocardial salvage index (0.56 vs 0.43,  $p=0.002$ ) (Table 3.6, Figure 3.10). There was also evidence of a reduction in both the presence (37% vs 72.4%,  $p=0.02$ ) and median amount (1.0 vs 4.5G,  $p=0.002$ ) of microvascular obstruction in the nitrite treated patients (Table 3.6).

TIMI $\leq 1$			
	Nitrite	Placebo	P value
<b>Baseline CMR</b>	<b>(n=27)</b>	<b>(n=30)</b>	
LVEDVi	75.69 (69.93-81.44)	70.33 (64.31-76.34)	0.19
LVESVi	35.16 (31.20-39.12)	35.41 (30.62-40.20)	0.94
LVMi	61.07 (56.27-65.88)	58.07 (54.44-61.71)	0.31
LVEF	53.86 (50.40-57.32)	50.00 (46.43-53.57)	0.12
Infarct size (%)	15.31 (12.36-18.27)	20.08 (16.72-23.43)	0.03
AAR	33.89 (30.54-37.24)	33.27 (29.12-37.42)	0.82
MSI	0.56 (0.50-0.62)	0.43 (0.37-0.49)	0.002
MVO			
No. (%)	10 (37.0%)	21 (72.4%)	0.02
Amount (median (IQR))	1.00 (0.80-5.87)	4.50 (1-7.50)	0.003
<b>6 month CMR</b>	<b>(n=25)</b>	<b>(n=29)</b>	
LVEDVi	79.27 (72.01-86.54)	75.37 (69.20-81.53)	0.40
LVESVi	33.80 (30.08-37.54)	34.94 (30.16-39.73)	0.71
LVMi	54.21 (50.35-58.07)	51.09 (47.75-54.44)	0.21
LVEF	57.19 (54.12-60.27)	54.51 (50.96-58.05)	0.25
Infarct size (%)	10.69 (8.38-13.02)	13.70 (11.16-16.24)	0.08

**Table 3.6 CMR data for study population with TIMI  $\leq 1$  flow at presentation.**

Values shown as mean (95% CI) unless otherwise stated. Abbreviations: LVEDVi, Indexed left ventricle end-diastolic volume; LVESVi, Indexed left ventricle end-systolic volume;

LVMi, Indexed left ventricle mass; LVEF, left ventricle ejection fraction; AAR, area at risk; MSI, myocardial salvage index; MVO, microvascular obstruction.



**Figure 3.10 Assessment of myocardial salvage index in the TIMI  $\leq 1$  patients on CMR.**

The size of the myocardial salvage index on CMR is presented for 28 TIMI  $\leq 1$  patients in the control group and 27 patients in the nitrite group. The mean index as assessed by CMR was significantly larger in the nitrite group compared to the control group ( $P=0.002$ ). Significance evaluated using unpaired t test and data shown as mean  $\pm$  SEM.

There was no evidence of difference in infarct size, area at risk, MSI or microvascular obstruction seen in patients with TIMI flow  $>1$  treated with nitrite compared to placebo (Table 3.7).

TIMI >1			
	Nitrite	Placebo	P value
<b>Baseline CMR</b>	<b>(n=4)</b>	<b>(n=5)</b>	
LVEDVi	78.01 (51.65-104.40)	71.99 (54.30-89.68)	0.58
LVESVi	39.90 (18.85-60.94)	37.37 (12.14-62.80)	0.84
LVMi	74.11 (58.19-90.03)	59.12 (40.75-77.49)	0.13
LVEF	49.55 (40.32-58.78)	50.50 (29.96-71.04)	0.92
Infarct size (%)	21.43 (9.62-33.23)	15.86 (0.42-31.30)	0.40
AAR	35.71 (21.15-50.28)	31.74 (21.62-41.87)	0.51
MSI	0.41 (0.31-0.51)	0.54 (0.30-0.78)	0.17
MVO			
No. (%)	3 (75.0%)	1 (25.0%)	0.14
Amount (median (IQR))	1 (0-8.25)	1 (0-1)	0.09
<b>6 month CMR</b>	<b>(n=2)</b>	<b>(n=4)</b>	
LVEDVi	109.94 (-184.6-404.5)	80.57 (60.05-101.1)	0.16
LVESVi	64.97 (-216.3-346.2)	37.42 (25.62-49.21)	0.13
LVMi	73.36 (-80.60-227.3)	52.14 (46.98-57.30)	0.05
LVEF	42.43 (-61.45-146.30)	53.51 (43.38-63.65)	0.18
Infarct size (%)	16.33 (-9.40-42.06)	10.59 (0.24-20.93)	0.32

**Table 3.7. CMR data for study population with TIMI ≤1 flow at presentation.**

Values shown as mean (95% CI) unless otherwise stated. Abbreviations: LVEDVi, Indexed left ventricle end-diastolic volume; LVESVi, Indexed left ventricle end-systolic volume; LVMi, Indexed left ventricle mass; LVEF, left ventricle ejection fraction; AAR, area at risk; MSI, myocardial salvage index; MVO, microvascular obstruction.

### **3.3.6 Acute safety and tolerability of nitrite across the whole cohort**

Following the administration of intra-coronary nitrite, 18 (45%) patients developed a >10% decrease in systolic blood pressure (within ten minutes) with a median decrease of 8.8mmHg (IQR: 0.5-30.1) however the magnitude and incidence was similar to the control group (23 patients, 57.5%, median drop 11.0 IQR: 2.1-21.4mmHg) (Figure 3.11). The drop in blood pressure did not alter clinical management. Whilst there was a small (but clinically insignificant) rise in met-Hb in the patients receiving nitrite (median 0.1% IQR: 0.0-0.13) this was not different to the control (median 0.0 IQR: 0-0.2%). No patients in the nitrite group developed sustained ventricular arrhythmias (either ventricular tachycardia or ventricular fibrillation) during the infusion of the drug, nor in the 10 minutes following the infusion.

### **3.3.7 Adverse events across the whole cohort**

During the first 48 hours after reperfusion, 7 adverse clinical events (17.5%) were recorded in the control group: 3 patients developed a >25% increase in creatine (CIN), 3 had episodes of heart failure and one had an episode of recurrent ischaemia which required further angiographic assessment. This compared to three adverse clinical events (7.5%) in the nitrite group: 1 episode of > 25% increase in creatine and 2 episodes of heart failure (p=0.311).



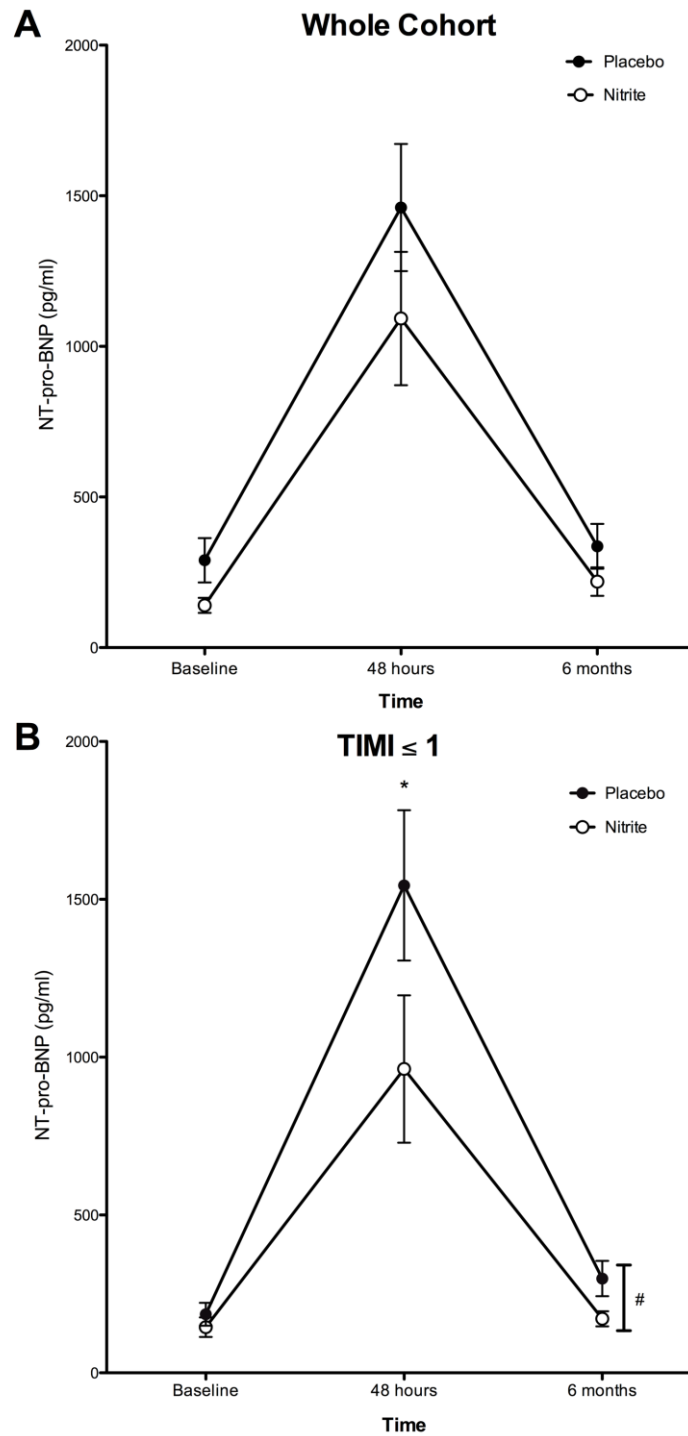


### **3.3.8 N-terminal pro-brain natriuretic peptide**

Blood analysis for N-terminal pro-brain natriuretic peptide (NT-proBNP) was performed on samples at baseline, 48 hours and 6 months for each patient. In both the nitrite and placebo groups, NT-pro BNP levels increased from baseline to 48 hours and then returned to normal levels at 6 months. There was no difference in the levels of NT-proBNP in the nitrite group versus the placebo group over the time period ( $p=0.12$ , Figure 3.11A). However there was a significant decrease in NT-proBNP levels in the TIMI  $\leq 1$  group with the biggest difference seen at 48 hours ( $p=0.01$ , Figure 3.11B). Levels of NT-proBNP were shown to correlate with both LVEF ( $p=0.001$ ,  $r=-0.401$ , Figure 3.12) and infarct size (CMR assessed Infarct size,  $p=0.0001$ ,  $r=0.464$ , cardiac enzyme assessed infarct size,  $p=0.0001$ ,  $r=0.404$  Figure 3.13)

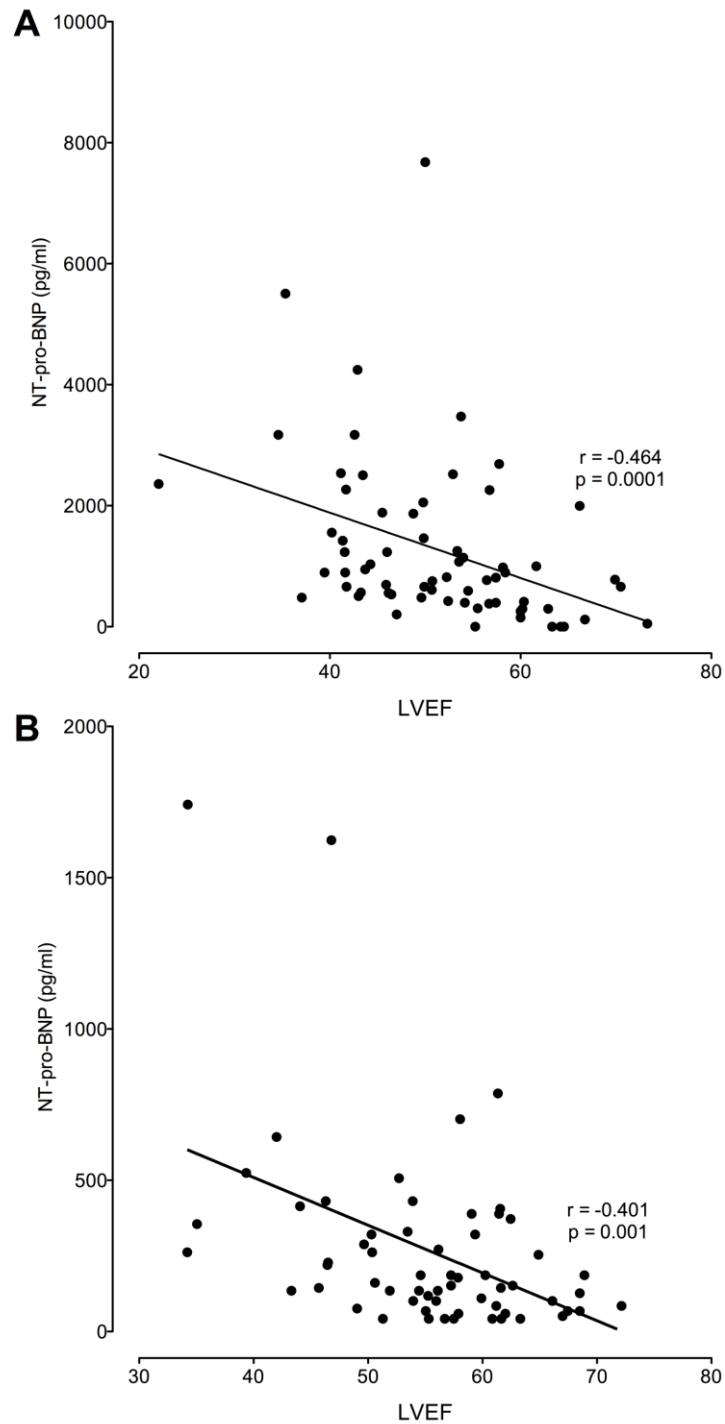
### **3.3.9 Medication**

Data was available on prescribed medication for 100% of patients at discharge, 97.5% at 6 months and 90% at 1 year. Overall the majority of both patient groups were optimised on medical therapy at discharge, 6 months and 1 year (dual anti-platelet agents, statin, beta-blocker and angiotensin-receptor blocking drugs). There were no differences in the prescription of medication between the treatment groups at discharge, 6 months or 1 year of follow-up (Table 3.8). No patient in either group underwent the insertion of cardiac devices (implantable cardiac defibrillator or cardiac resynchronization therapy) during the 1<sup>st</sup> year of follow-up.



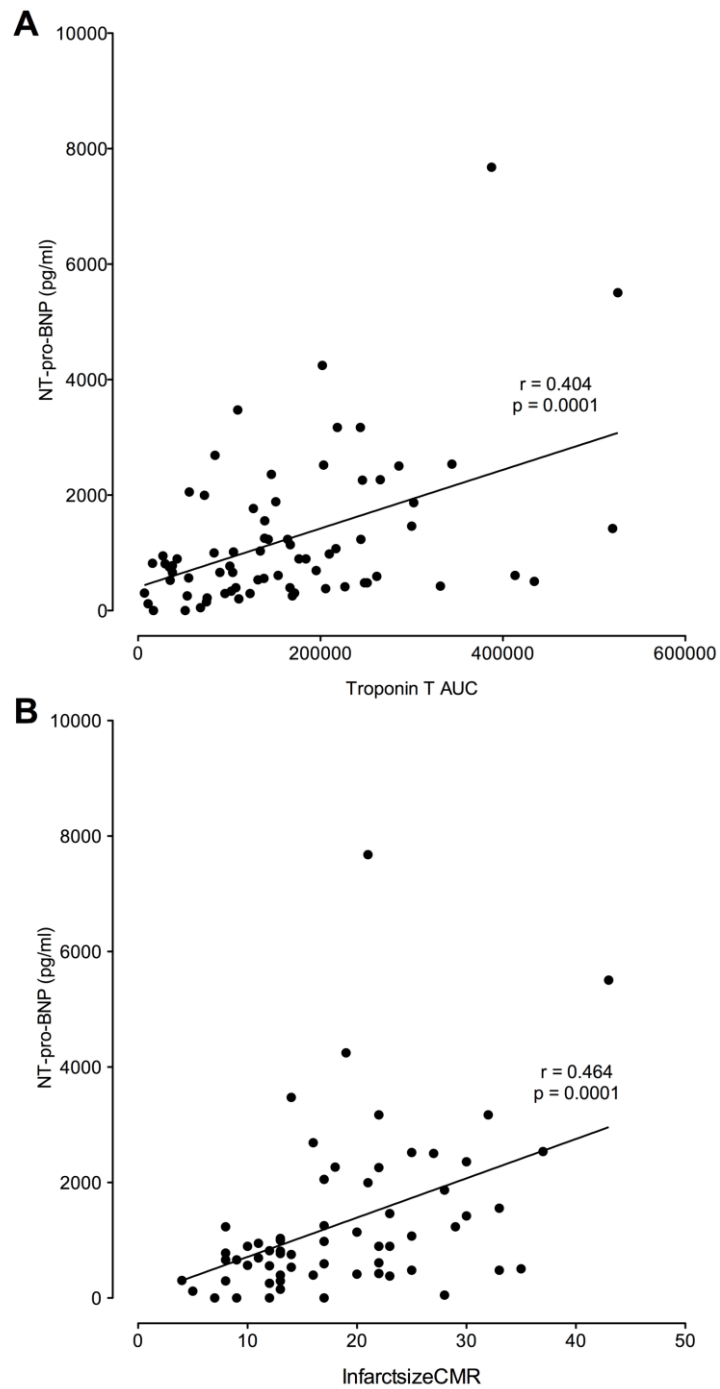
**Figure 3.11 NT-proBNP and treatment group.**

NT-proBNP levels were measured at baseline, 48 hours, and 6 months after coronary reperfusion. NT-proBNP levels over time for nitrite versus placebo for all patients are shown in panel A. Panel B shows NT-proBNP levels in patients with TIMI flow <1. Data expressed as mean  $\pm$  SEM. #= $P$ <0.05, for two-way repeated measures ANOVA, \* for  $p$ <0.05 using two-way repeated measures ANOVA with Bonferroni's multiple comparison test.



**Figure 3.12 Associations between NT-proBNP and left-ventricular ejection fraction (LVEF).**

There was a significant negative correlation between NT-proBNP levels at 48 hours and LVEF assessed by CMR at 48 hours ( $r=-0.464$ ), as shown in panel A. Panel B depicts a similar negative association between the NT-proBNP levels at 6 months and LVEF assessed by CMR ( $r=-0.401$ ) at the same time-point. Associations determined using Spearman's correlation coefficient assessment.



**Figure 3.13 Associations between NT-proBNP and infarct size.**

There was a significant positive correlation between NT-proBNP levels at 48 hours and infarct size as assessed by Troponin T AUC ( $r=0.404$ ), as shown in panel A. Panel B depicts a similar positive association between the NT-proBNP levels at 48 hours and infarct size assessed by late-gadolinium enhancement on CMR ( $r=0.464$ ). Associations determined using Spearman's correlation coefficient assessment.

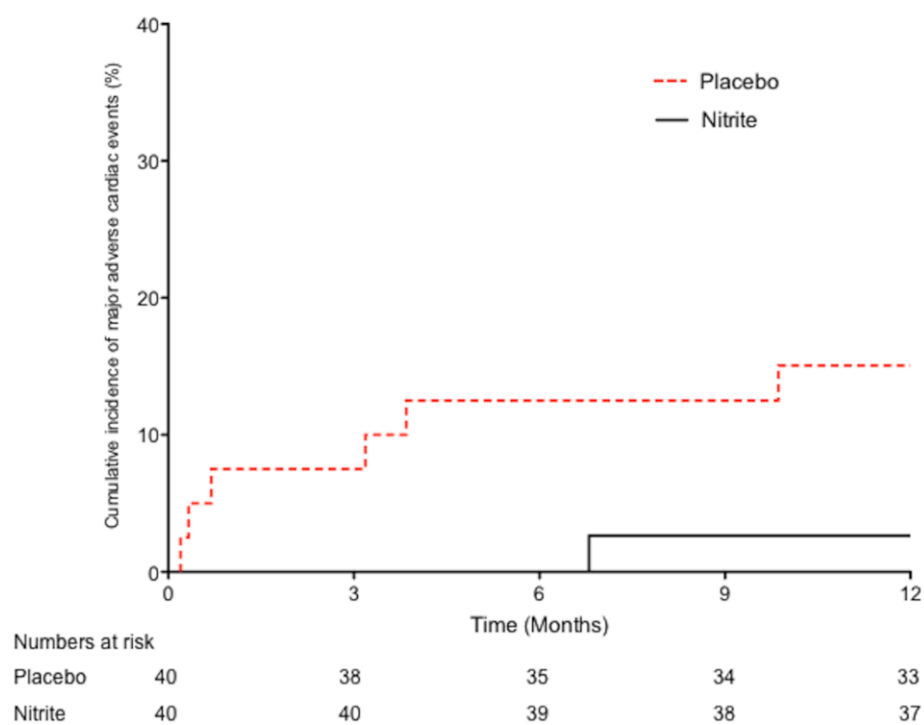
	Nitrite	Placebo	P value
<b>Medication at discharge</b>	<b>(n=40)</b>	<b>(n=40)</b>	
ACE inhibitor	39 (97.5%)	36 (90.0%)	0.359
ARB	1 (2.5%)	2 (5.0%)	0.556
Diuretic	2 (5.0%)	4 (10.0%)	0.396
Ivabradine	1 (2.5%)	0 (0%)	0.314
β Blocker	37 (92.5%)	36 (90.0%)	0.692
Statin	40 (100%)	40 (100%)	1.000
Aspirin	40 (100%)	40 (100%)	1.000
Clopidogrel	37 (92.5%)	37 (92.5%)	1.000
Prasugrel	3 (7.5%)	2 (5.0%)	0.644
Organic Nitrate	0 (0%)	1 (2.5%)	0.314
<b>Medication at 6 months</b>	<b>n=39</b>	<b>n=39</b>	
ACE inhibitor	36 (92.3%)	34 (87.2%)	0.455
ARB	3 (7.7%)	4 (10.3%)	0.692
Diuretic	2 (5.1%)	2 (5.1%)	1.000
Spironolactone	0 (0.0%)	1 (100)	0.314
Ivabradine	2 (5.1%)	0 (0.0%)	0.147
β Blocker	36 (92.3%)	34 (87.2%)	0.455
Statin	39 (100%)	39 (100%)	1.000
Aspirin	39 (100%)	39 (100%)	1.000
Clopidogrel	35 (89.7%)	35 (89.7%)	1.000
Prasugrel	3 (7.7%)	2 (5.1%)	0.644
Organic Nitrate	0 (0.0%)	1 (2.6%)	0.314
<b>Medication at 1 year</b>	<b>n=36</b>	<b>n=36</b>	
ACE inhibitor	33 (91.7%)	31 (86.1%)	0.453
ARB	3 (8.3%)	5 (13.9%)	0.453
Diuretic	1 (2.8%)	1 (2.8%)	1.000
Spironolactone	0 (0.0%)	1 (2.8%)	0.314
Ivabradine	2 (5.6%)	0 (0.0%)	0.152
β Blocker	33 (91.7%)	32 (88.9%)	0.691
Statin	36 (100.0%)	36 (100.0%)	1.000
Aspirin	36 (100.0%)	35 (97.2%)	0.314
Clopidogrel	27 (75.0%)	27 (75.0%)	1.000
Prasugrel	3 (8.3%)	2 (5.6%)	0.643
Nitrate	0 (0.0%)	1 (2.8%)	0.314

**Table 3.8 Prescribed drugs by study treatment.**

Values shown as number (%) unless otherwise stated. Abbreviations: ACEi: Angiotension-converting enzyme; ARB: Angiotensin receptor blocker.

### 3.3.10 Major adverse cardiac events

1 year after infarction, six patients (15.8%) in the control group suffered MACE (2 hospitalisations for heart failure, 1 recurrent myocardial infarction and 3 unscheduled revascularizations) compared to 1 patient (2.6%) (1 unscheduled revascularization) in the nitrite group ( $p=0.04$ ) (Figure 3.14).



**Figure 3.14. Kaplan Meier curves showing cumulative probability of major adverse cardiac events (MACE) after primary PCI according to treatment group.**

### 3.4 Summary

1. Intra-coronary nitrite infusion appears safe in patients undergoing primary PCI for AMI.
2. In the whole trial population, nitrite infusion was not associated with a reduction in infarct size as assessed by cardiac enzymes (CK or Troponin T) or CMR imaging.
3. Despite the negative primary endpoint there was a suggestion of benefit associated with nitrite in the whole trial population with a trend to improvement in the myocardial salvage index on CMR and a significant reduction in 1 year MACE in the nitrite treated group
4. In a single sub-group analysis splitting the trial population by TIMI flow count, nitrite was associated with a significant reduction in infarct size assessed by CK in patients with TIMI flow  $\leq 1$ . In this sub-group, nitrite was also associated with a significant reduction in CMR assessed infarct size and microvascular obstruction and a significant increase in the myocardial salvage index.
5. No difference in infarct size was seen with nitrite treatment in patients with TIMI flow  $>1$



# **Chapter 4.0**

## **Investigation of the potential mechanisms by which nitrite reduces I/R injury**

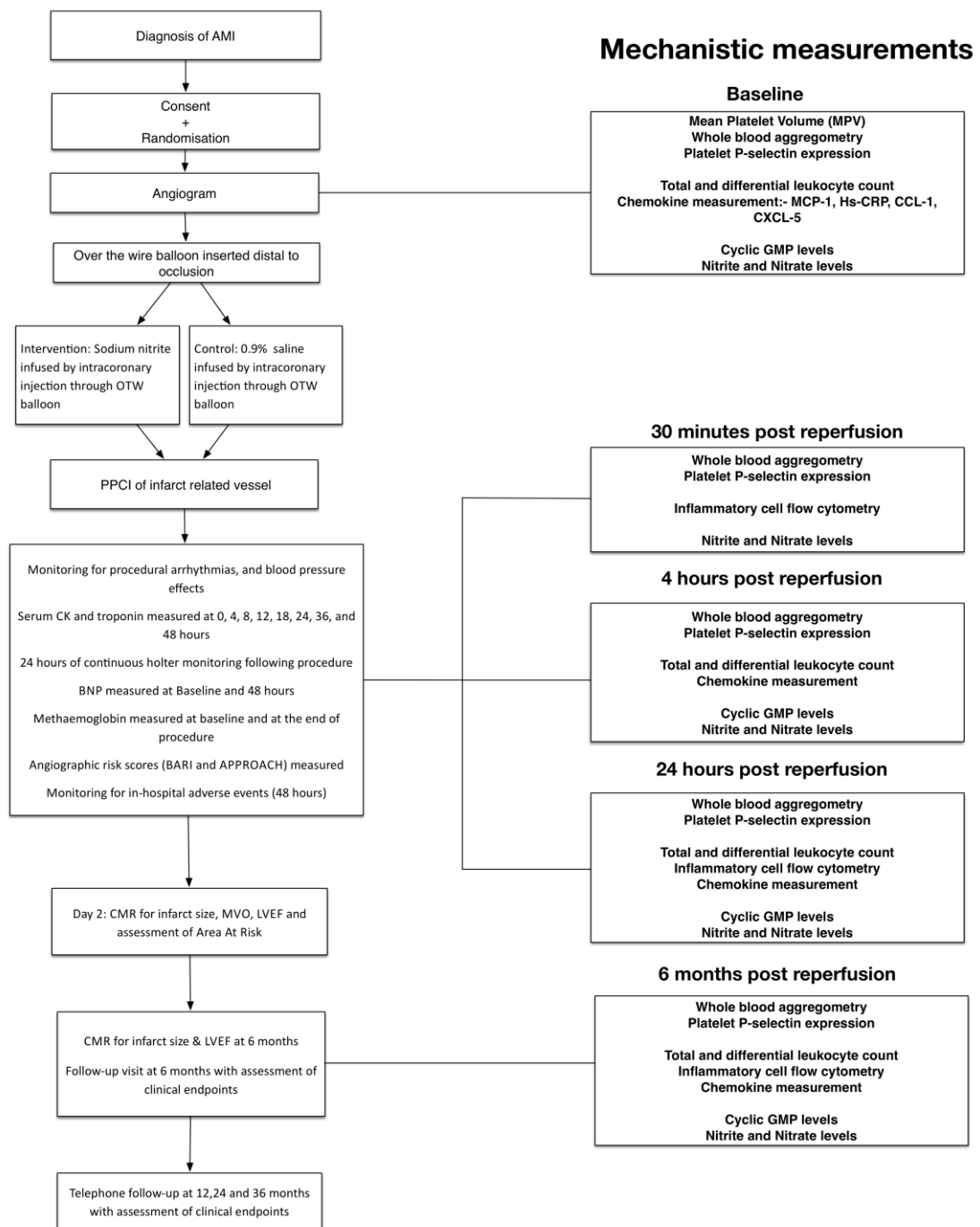
## 4.1 Introduction

Nitrite has been shown in pre-clinical studies to be cardioprotective and reduce myocardial infarct size, however the exact mechanisms by which this benefit is conferred are not clear. It is clear however that the beneficial effects of nitrite are due to its conversion to NO, as evidenced by the complete block of nitrite-induced protection by the NO scavenger carboxy-PTIO, (Webb et al., 2004; Duranski MR et al., 2005). NO itself exerts a number of actions (Moncada, Higgs 1993) that are thought to be cardioprotective (Johnson 1991). In particular, NO plays an important role in modulating many aspects of the inflammatory response. Indeed evidence demonstrates that NO inhibits leukocyte recruitment (Kubes et al., 1991) via a cGMP dependent manner (Ahluwalia et al., 2004), a component of the damage caused in I/R injury. Evidence has suggested that reducing the inflammatory cell-driven injury to the endothelium and perivascular myocardium (dPalazzo AJ et al., 1998) results in a positive outcome in I/R injury. Platelet adhesion and aggregation are also important and are well recognized as terminal events in the formation of thromboses leading to MI (Davi G, Patrono, C 2007). It is widely accepted that NO suppresses the adhesion of platelets to intact vascular endothelium (Radomski et al, 1987) and also platelet aggregation itself (Radomski et al, 1990). These effects as with inflammatory cell recruitment are also mediated by cGMP. Interestingly a further common pathway of both NO-mediated suppression of cell recruitment and platelet reactivity is a cGMP-dependent suppression of P-selectin expression. It is therefore important to establish whether nitrite has effects on both platelet reactivity and inflammation when given intra-coronary during primary PCI.

Thus the aim of the study described in this chapter is to assess the mechanisms by which intra-coronary nitrite reduces reperfusion injury following primary PCI in patients with STEMI, specifically focusing on platelet activity and inflammation as per the study design described in section 2.1 and chapter 3.

#### **4.2 Studies for platelet reactivity and inflammatory markers**

The study was powered for infarct size however whether there was sufficient power for the assessment of platelet reactivity and inflammatory markers is unknown. However the aim of this part of the thesis was primarily for hypothesis generation. As per figure 4.1 and described in chapter 2, a number of assessments based upon platelet function and inflammatory cell recruitment and circulating mediators were conducted (Figure 4.1).



**Figure 4.1 Flow diagram of Study design and mechanistic measurement points**

AMI: acute myocardial infarction, OTW: Over the wire balloon, PPCI: primary percutaneous coronary intervention, CMR: cardiac magnetic resonance imaging, MVO: microvascular obstruction, LVEF: Left ventricular ejection fraction

## 4.3 Results

### 4.3.1 Effect of intra-coronary nitrite on platelet reactivity post-primary PCI

#### 4.3.1.1 Baseline Platelet measures

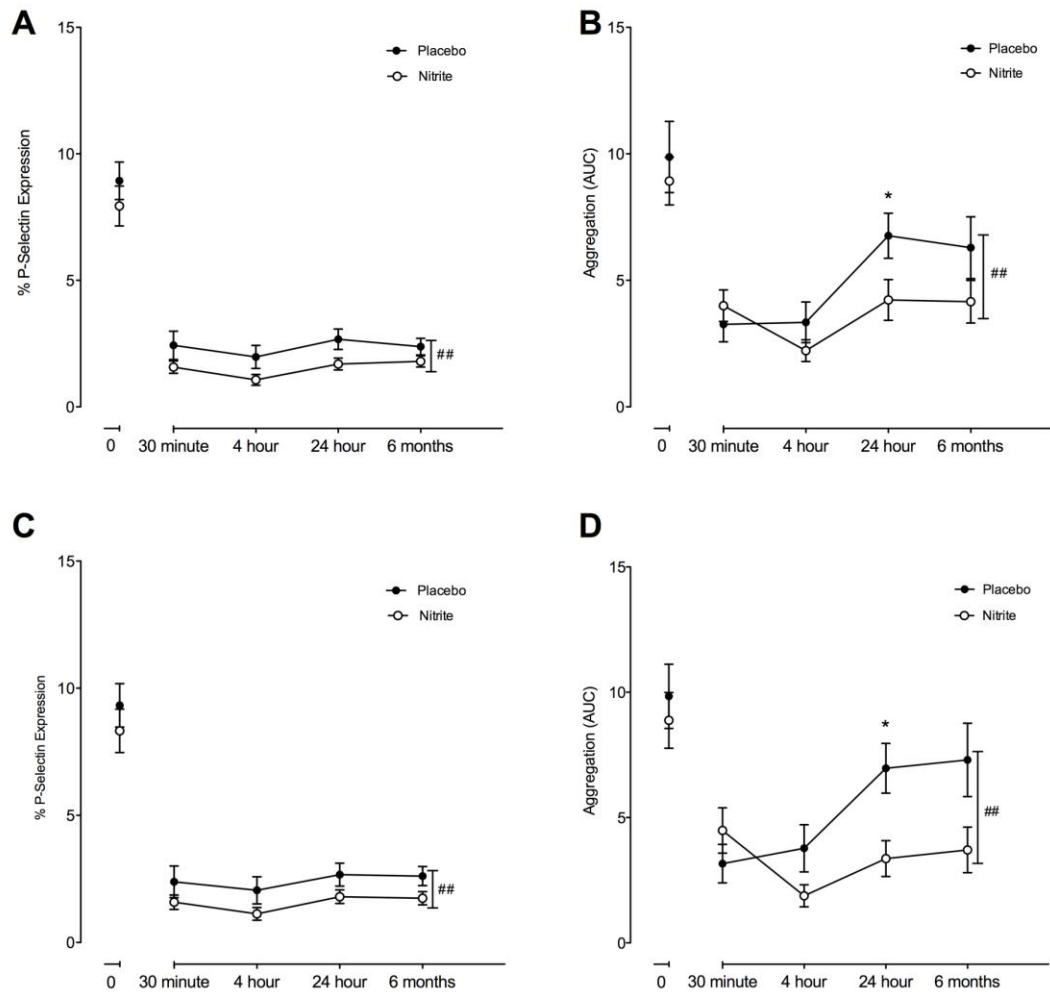
The nitrite and placebo groups were similar with respect to all measures of platelet function and reactivity at baseline (Table 4.1).

	Nitrite	Placebo	P value
<b>Platelet Count (x10<sup>9</sup>/L)</b>	<b>245.6±8.8</b>	<b>262±11.7</b>	<b>0.264</b>
<b>Platelet Volume (fL)</b>	<b>10.8±0.2</b>	<b>11.1±0.2</b>	<b>0.256</b>
<b>P-selectin expression (%)</b>	<b>8.05±0.77</b>	<b>9.8±0.93</b>	<b>0.153</b>
<b>WBA (AUC)</b>	<b>8.93±0.95</b>	<b>9.88±1.41</b>	<b>0.577</b>

**Table 4.1 Baseline platelet characteristics of the study population**

All measures were performed in 40 nitrite and 40 placebo patients aside from mean platelet volume, which was measured in 30 nitrite and 30 placebo patients. P-Selectin expression and Whole Blood Aggrerometry (WBA) measured in response to control (PBS). Values expressed as mean ± SEM. Statistical comparisons performed using unpaired T test.

Following reperfusion both P-selectin expression and spontaneous aggregation (PBS control) changed over time in both the whole cohort (Figure 4.2A and B) and the TIMI <1 cohort (Figure 4.2C and D).

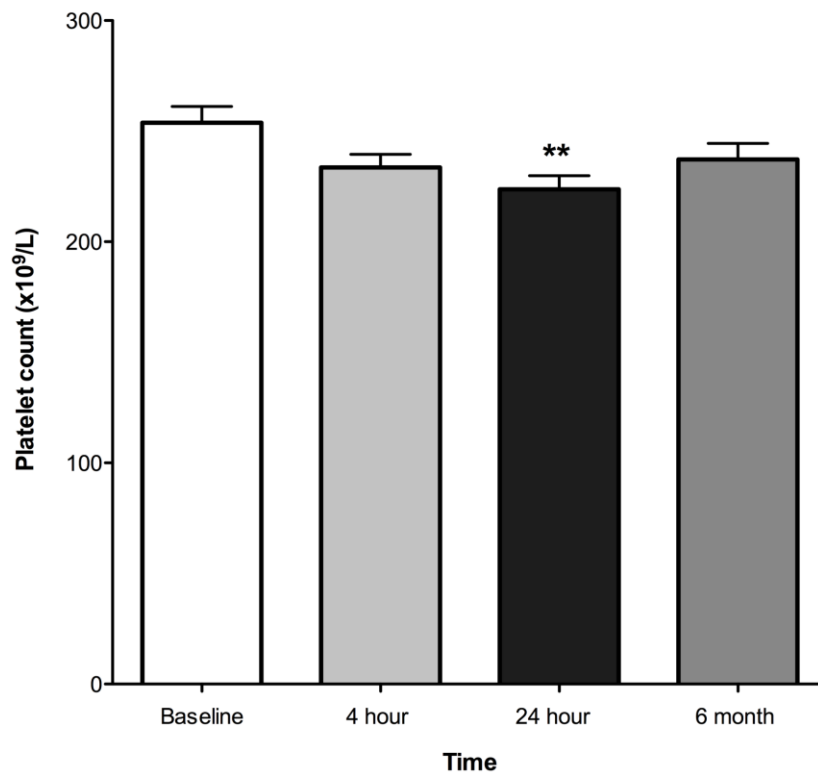


**Figure 4.2. P-selectin expression and spontaneous aggregation over time (Control)**

Platelet reactivity measured at baseline, 30 minutes, 4 hours, 24 hours and 6 months after coronary reperfusion. Platelet P-Selectin expression assessed in whole blood following incubation with phosphate buffered saline control (PBS) is shown for nitrite versus placebo for all patients in panel A. Panel B show whole, blood impedance aggregometry in response to the same PBS stimulus in all patients. Panel C shows P-selectin expression in response to PBS in patients with TIMI flow <1. Panel D shows aggregation in response to PBS in the TIMI <1 subgroup. All panels show nitrite treated versus placebo. Data expressed as mean  $\pm$  SEM. # =  $P < 0.05$ , ## =  $P < 0.01$ , for two-way repeated measures ANOVA. \*  $p < 0.05$  using two-way repeated measures ANOVA with Bonferroni's multiple comparison test. PBS=Phosphate buffered saline.

#### 4.3.1.2 Platelet count over time post-primary PCI

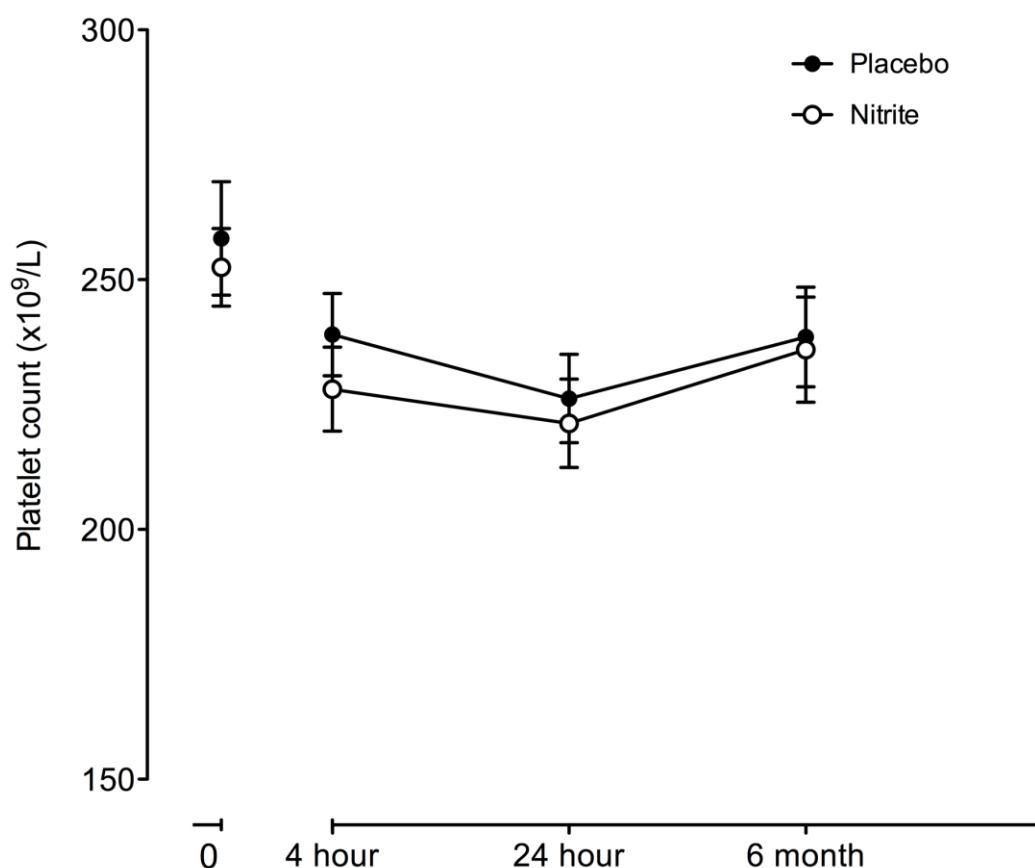
Total platelet counts were measured at baseline, 4 hours, 24 hours and 6 months after coronary reperfusion. In all patients platelet levels were at their highest at baseline ( $253.8 \pm 7.3 \times 10^9$ ) with a significant change over time with levels decreasing at 4 hours and the lowest levels seen at 24 hours compared to baseline ( $223.7 \pm 6.2 \times 10^9$  versus  $253.8 \pm 7.3 \times 10^9$ ,  $P=0.002$ ). Levels at 6 months were similar to but not back to baseline levels ( $237.2 \pm 7.2 \times 10^9$  versus  $253.8 \pm 7.3 \times 10^9$ ) (Figure 4.3).



**Figure 4.3 Platelet count over time post primary PCI**

Total platelet count measured at baseline, 4 hours, 24 hours and 6 months after coronary reperfusion. Platelet count assessed over time in all patients is shown. Data expressed as mean  $\pm$  SEM. \*\* $P<0.01$  using 1-way ANOVA, with Bonferroni's multiple comparison test.

No difference was seen in total platelet counts when splitting patients into nitrite and control groups after coronary reperfusion ( $p=0.360$ ) (Figure 4.4).



**Figure 4.4 Platelet count over time by treatment group after primary PCI.**

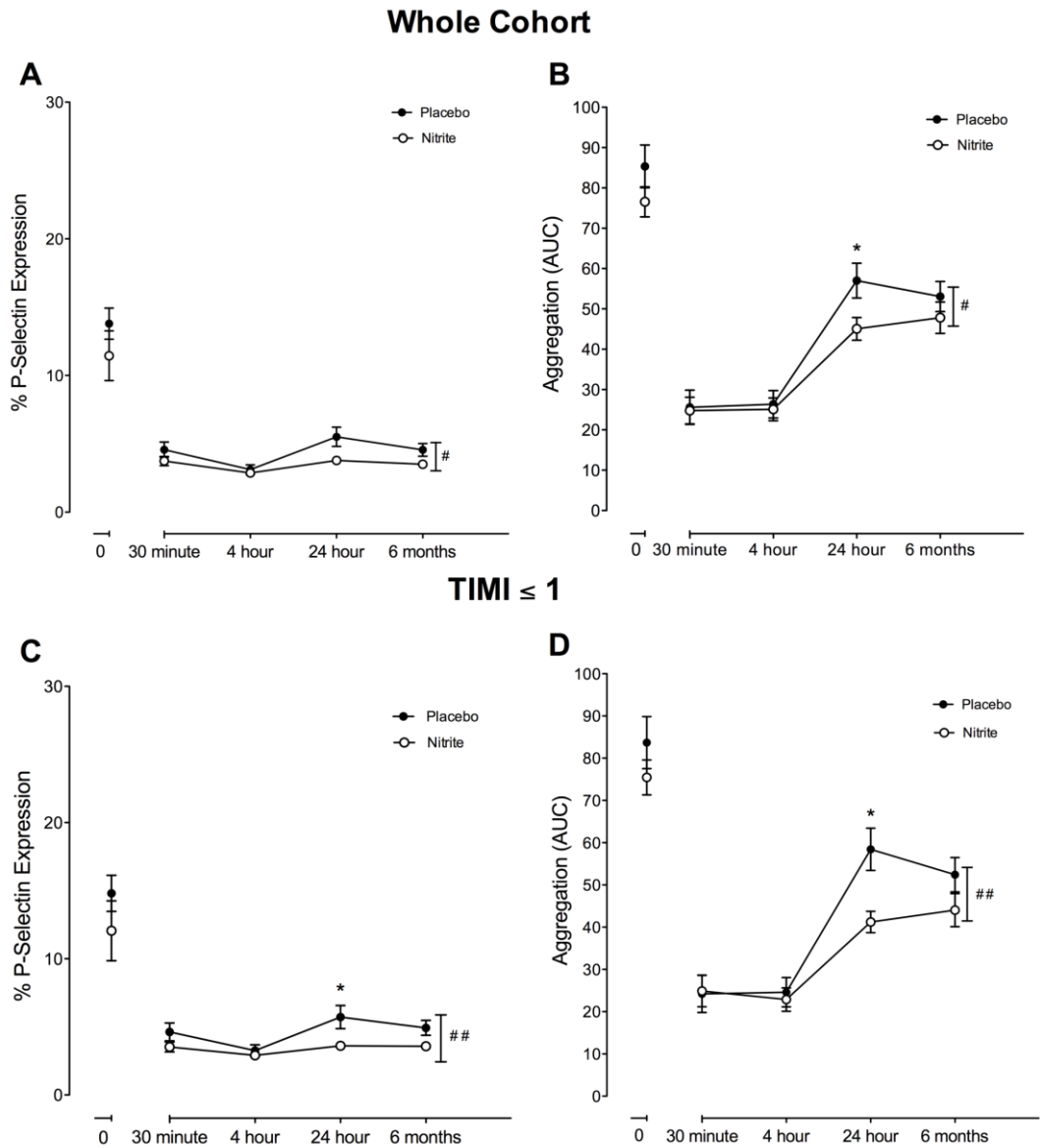
Total platelet count measured at baseline, 4 hours, 24 hours and 6 months after coronary reperfusion. Figure displays platelet count over time showing nitrite treated versus placebo. Data expressed as mean  $\pm$  SEM. Statistical comparison performed using two-way repeated measures ANOVA.



#### **4.3.1.3 Platelet reactivity in response to collagen pre and post primary PCI**

Platelet reactivity was assessed by whole blood aggregometry (WBA) and platelet P-selectin expression at baseline, 30 minutes, 4 hours, 24 hours and 6 months post reperfusion. Platelet aggregation and platelet P-selectin expression changed substantially over time in response to collagen (Figure 4.5) in both placebo and nitrite treated groups. The greatest response occurred at baseline with a decrease at 4 hours, followed by a slight elevation at 24 hours and a further decrease by 6 months. The response to collagen was similar at baseline between the two treatment groups, however, there was evidence of a decrease in aggregatory response in the nitrite group versus placebo as assessed by both P-selectin expression (Figure 4.5A) and platelet aggregation (Figure 4.5B) in response to collagen over time after PCI.

When looking at platelet reactivity in the TIMI <1 subgroup where a significant reduction in infarct size with nitrite was seen, these differences were still evident and statistically increased between the groups (Figure 4.5C and D).



**Figure 4.5 Platelet reactivity (Collagen).**

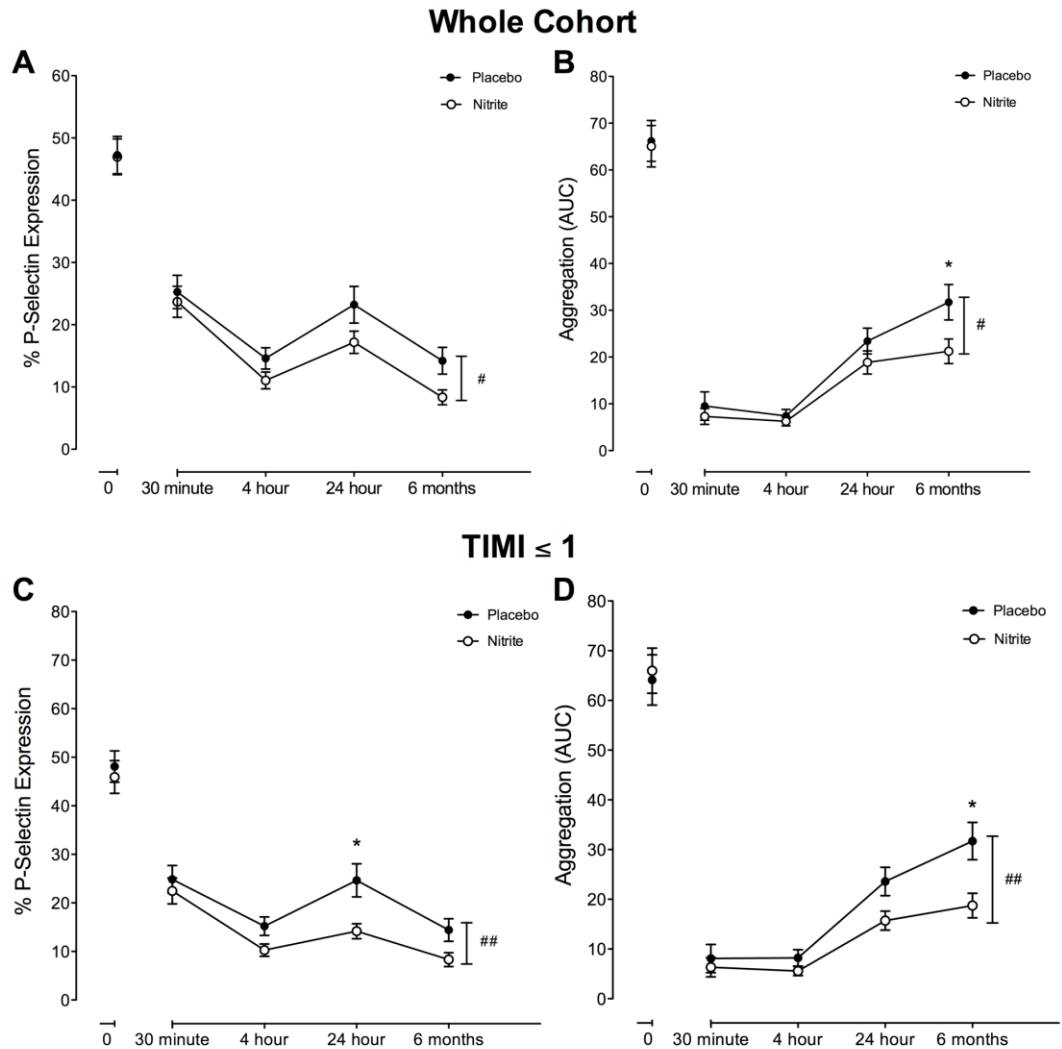
Platelet reactivity measured at baseline, 30 minutes, 4 hours, 24 hours and 6 months after coronary reperfusion. Platelet P-Selectin expression assessed in whole blood in response to collagen (3  $\mu$ mol/L) is shown for nitrite versus placebo for all patients in panel A. Panel B show using whole blood impedance aggregometry in response to the same collagen stimulus in all patients. Panel C shows P-selectin expression in response to collagen in patients with TIMI flow <1. Panel D shows aggregation in response to collagen in the TIMI <1 subgroup. All panels show nitrite treated versus placebo. Data expressed as mean  $\pm$  SEM. # =  $P < 0.05$ , ## =  $P < 0.01$ , for two-way repeated measures ANOVA, \* =  $p < 0.05$  using two-way repeated measures ANOVA with Bonferroni's multiple comparison test.

#### **4.3.1.3 Platelet reactivity in response to ADP pre and post primary PCI**

Platelet aggregation and platelet P-selectin expression changed substantially over time in response to ADP (Figure 4.6) in both placebo and nitrite treated groups. Similar to the response to collagen the greatest response occurred at baseline with a decrease at 4 hours, followed by a slight elevation at 24 hours and a further decrease by 6 months. Responses to ADP were similar at baseline between the two treatment groups, however, there was evidence of a decrease in aggregatory response in the nitrite group versus placebo as assessed by both P-selectin expression (Figure 4.6A) and platelet aggregation (Figure 4.6B) in response to ADP over time after primary PCI.

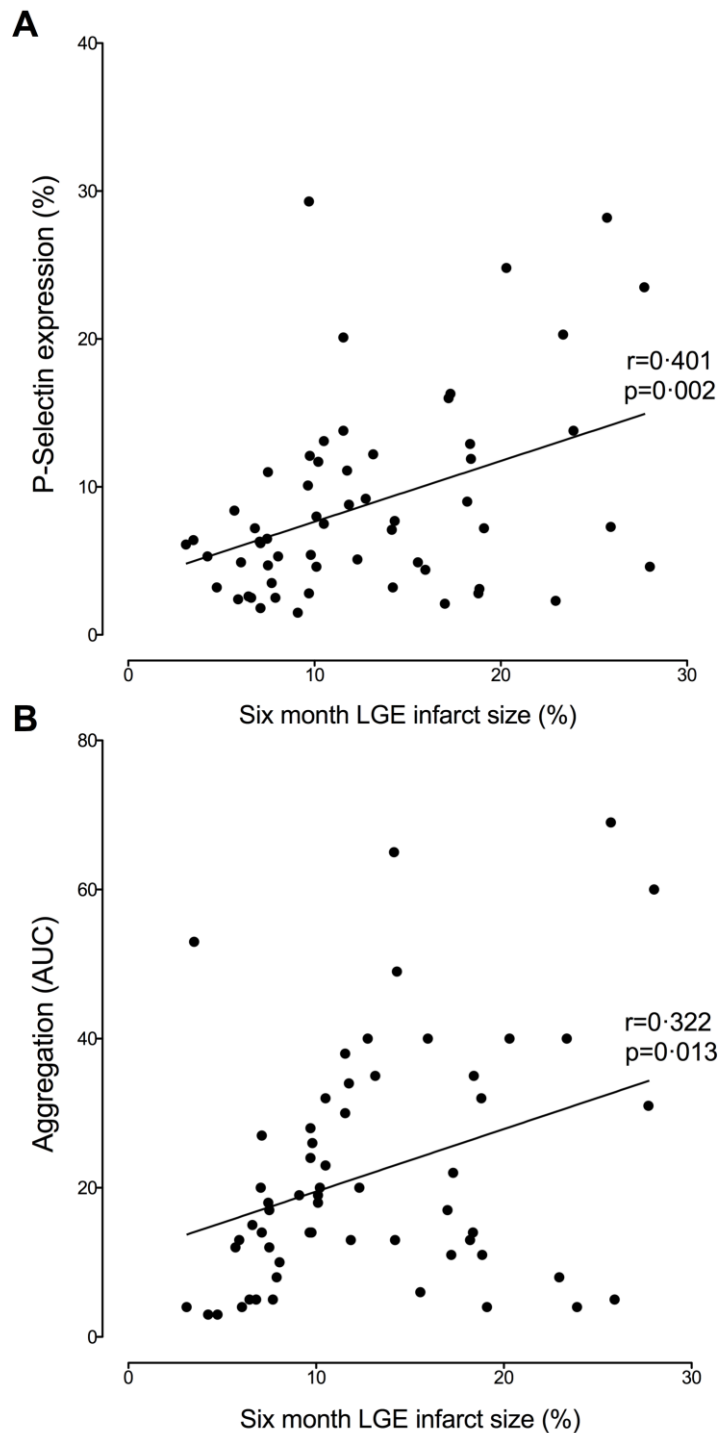
When looking at platelet reactivity in the TIMI <1 subgroup, these differences were still evident and statistically increased between the groups (Figure 4.6C and D).

Post-hoc analyses demonstrated that the reactivity of platelets was directly associated to CMR-determined infarct size (Figure 4.7). 6 month CMR infarct size was positively correlated with both P-selectin expression ( $r=0.401$ ,  $p=0.002$ ) and platelet aggregation ( $r=0.344$ ,  $p=0.007$ ) at 6 months in response to ADP.



**Figure 4.6 Platelet reactivity (ADP).**

Platelet reactivity measured at baseline, 30 minutes, 4 hours, 24 hours and 6 months after coronary reperfusion. Platelet P-Selectin expression assessed in whole blood in response to ADP (10  $\mu$ mol/L) is shown for nitrite versus placebo for all patients in panel A. Panel B shows whole blood impedance aggregometry in response to the same ADP stimulus in all patients. Panel C shows P-selectin expression in response to ADP in patients with TIMI flow <1. Panel D shows aggregation in response to ADP in the TIMI <1 subgroup. All panels show nitrite treated versus placebo. Data expressed as mean  $\pm$  SEM. # =  $P < 0.05$ , ## =  $P < 0.01$ , for two-way repeated measures ANOVA, \* =  $p < 0.05$  using two-way repeated measures ANOVA with Bonferroni's multiple comparison test. (ADP: adenosine diphosphate).



**Figure 4.7 Associations between platelet reactivity and infarct size on cardiac magnetic resonance imaging (CMR) at 6 months.**

There was a significant positive association between platelet P-selectin expression in response to ADP and LGE (late gadolinium enhancement) assessed infarct size on CMR (late gadolinium enhancement) at six months, as shown in panel A. Panel B depicts a similar positive association between platelet aggregation in response to ADP and LGE CMR infarct size at six months. Correlations determined using Pearson's correlation coefficient.

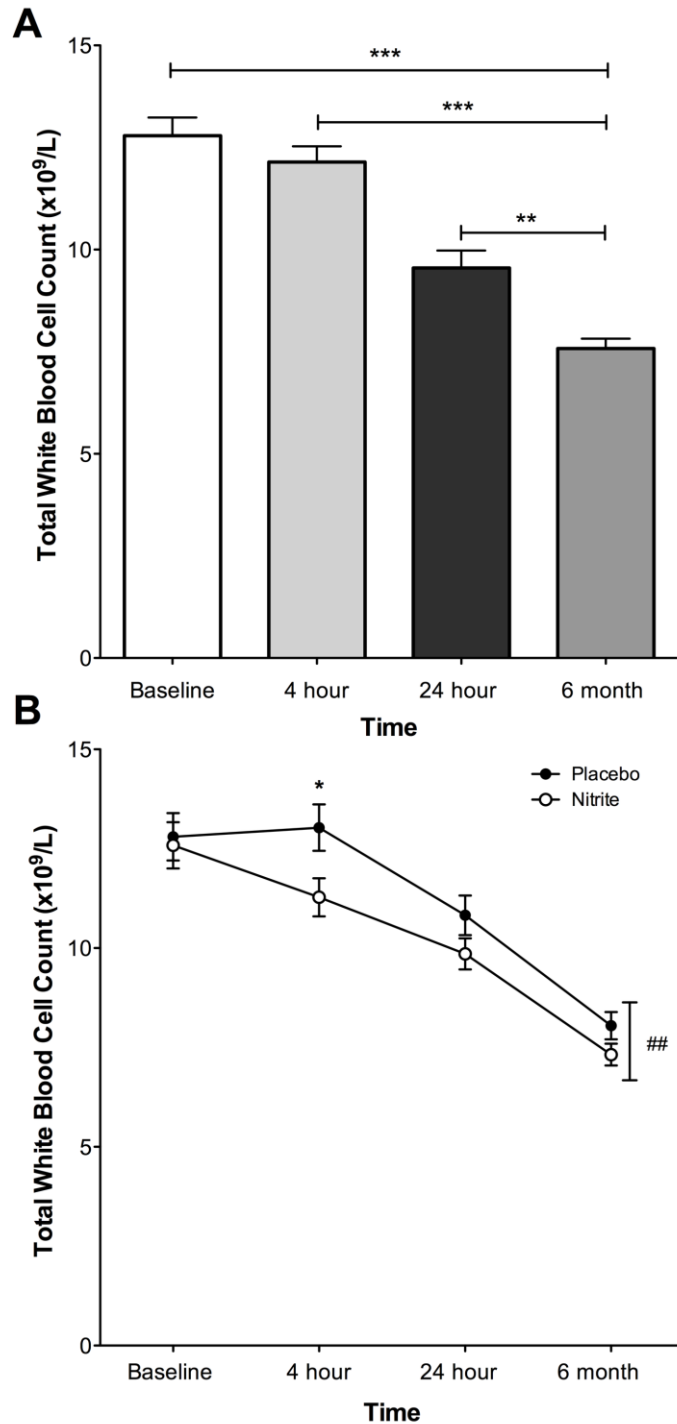
### **4.3.2 Effect of nitrite on the systemic inflammatory response post primary PCI**

#### **4.3.2.1 Total leukocyte cell count**

Total leukocyte cell counts were measured at baseline, 4 hours, 24 hours and 6 months after coronary reperfusion in all patients. Total leukocyte count changed significantly over time with highest levels seen at baseline, declining slightly at 4 hours with a further drop at 24 hours with the lowest levels seen at 6 months ( $P<0.0001$ ) (Figure 4.8A). Leukocyte cell counts were similar at baseline between the nitrite and placebo groups. However there was evidence of a decreased leukocyte count in the nitrite group compared to placebo group over time ( $P=0.0079$ ) (Figure 4.8B) with the largest difference seen at 4 hours.

#### **4.3.2.2 Differential leukocyte cell count**

Differential leukocyte counts (neutrophils, lymphocytes and monocytes) were measured at the same time-points as the total leukocyte cell count (baseline, 4 hours, 24 hours and 6 months). Neutrophil counts were high at baseline, increased slightly at 4 hours before declining significantly by 24 hours with the lowest levels seen at 6 months ( $p<0.0001$ ) (Figure 4.9A). Lymphocyte numbers were similar at baseline, 24 hours and 6 months, however significantly lower levels were seen at 4 hours ( $P<0.0001$ ) (Figure 4.9C).



**Figure 4.8 Total leukocyte count pre and post primary PCI.**

Total leukocyte count was measured at baseline, 4 hours, 24 hours and 6 months after coronary reperfusion. Leukocyte count assessed over time in all patients is shown in panel A. Panel B shows leukocyte cell count over time showing nitrite (n=40) treated versus placebo (n=40). Data expressed as mean  $\pm$  SEM. \*\*\*P<0.0001, \*\*P<0.01 using one-way ANOVA, with Bonferroni's multiple comparison test. ##=P<0.01, for two-way repeated measures ANOVA, \* for p<0.05 using two-way repeated measures ANOVA with Bonferroni's multiple comparison test.

Monocyte numbers were similar from baseline to 4 hours but increased significantly at 24 hours with a decline by 6 months ( $p<0.0001$ ) (Figure 4.9E).

All differential leukocyte cell counts were similar at baseline between the nitrite and placebo treatment groups. However, there was evidence of a decrease in the neutrophil cell count over time in the nitrite group versus placebo ( $p=0.0062$ , Figure 4.9B), with the largest difference seen at 4 hours. No difference was seen between either monocyte or total lymphocyte numbers between the 2 treatment groups over time (monocytes  $p=0.425$ , lymphocytes  $p=0.365$ ) (Figures 4.9D/F)

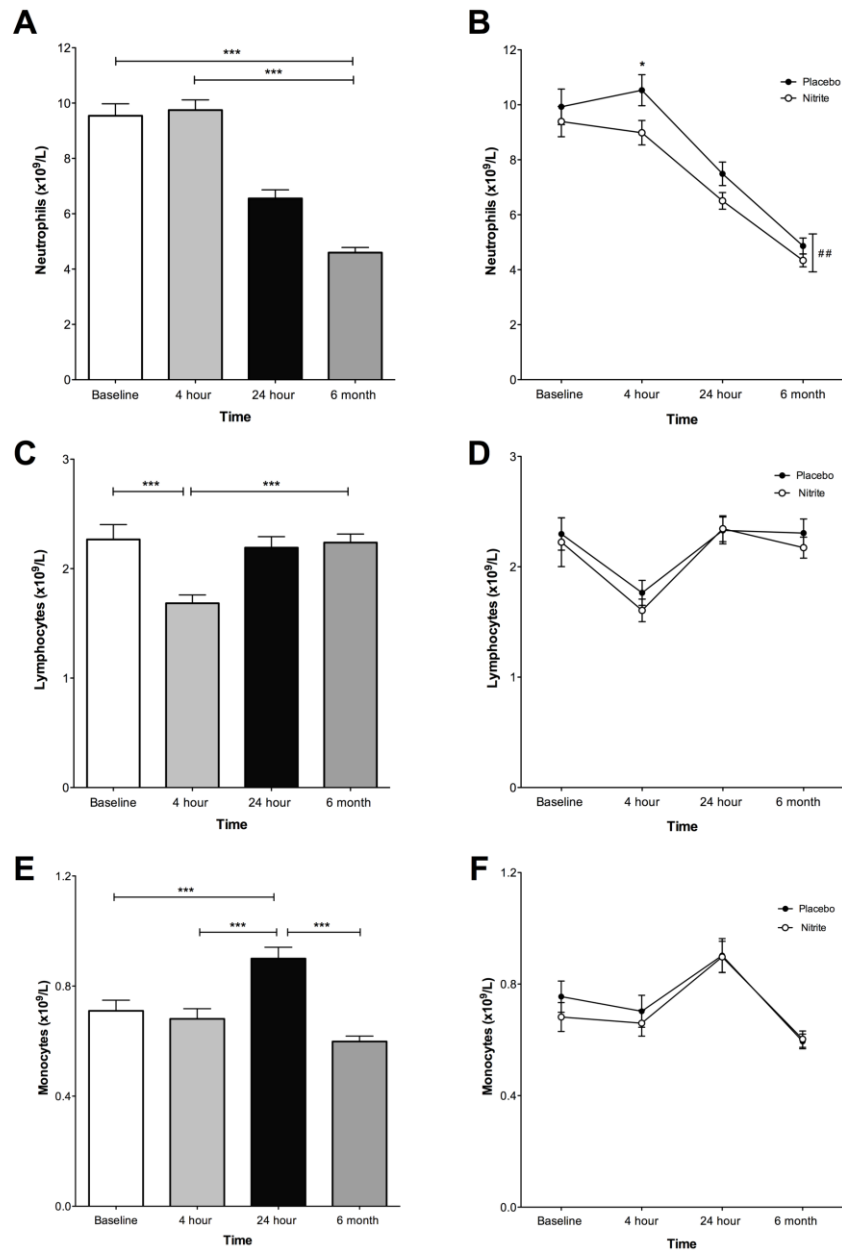
#### **4.3.2.3 Leukocyte inflammatory marker expression**

Inflammatory marker expression (CD11b, CD62L and CD162) on each leukocyte population: neutrophils, monocytes (inflammatory, intermediate and resident), and T lymphocytes (CD4<sup>+</sup> and CD8<sup>+</sup>) were measured. Measurements were made at 30 minutes, 24 hours and 6 months post reperfusion in 23 patients in the placebo group and 21 patients in the nitrite group.

#### **4.3.2.4 Neutrophil activation marker expression**

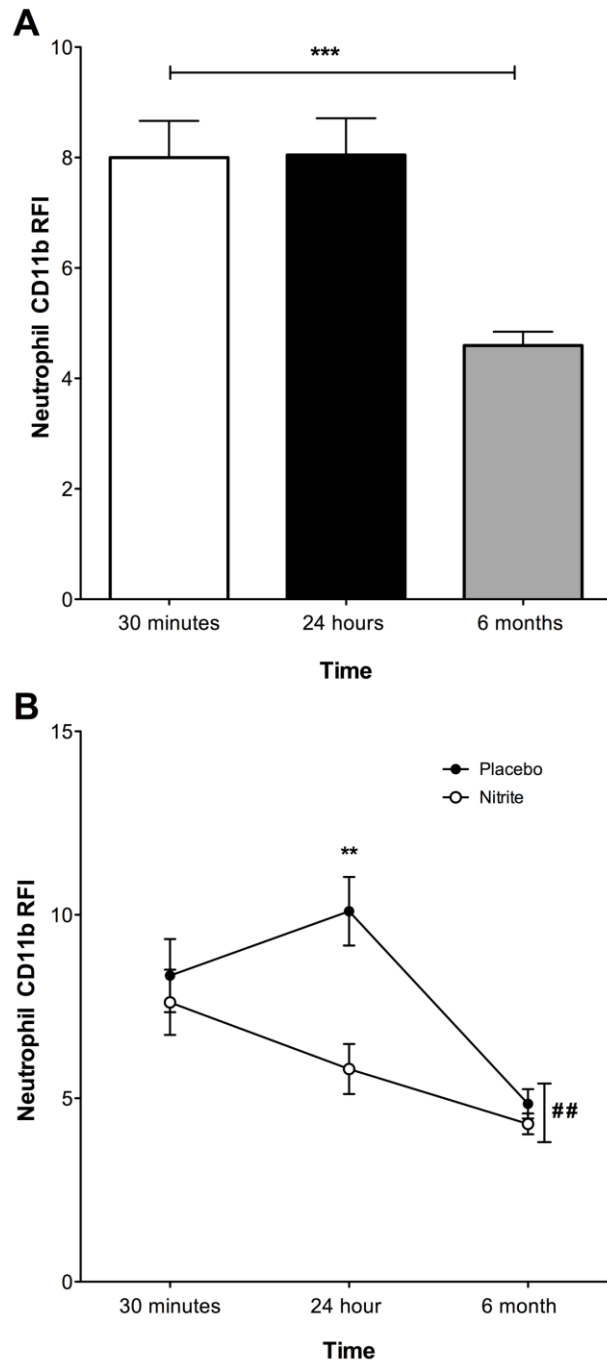
Neutrophil CD11b relative fluorescence intensity (RFI) was similar at 30 minutes and 24 hours post reperfusion with significantly lower levels seen at 6 months after reperfusion ( $p<0.0001$ ) (Figure 4.10A). CD11b RFI was significantly lower in the nitrite treated patients compared to placebo over the time-points with the greatest difference evident at 24 hours ( $p=0.0034$ ) (Figure 4.10B).





**Figure 4.9 Differential Leukocyte count.**

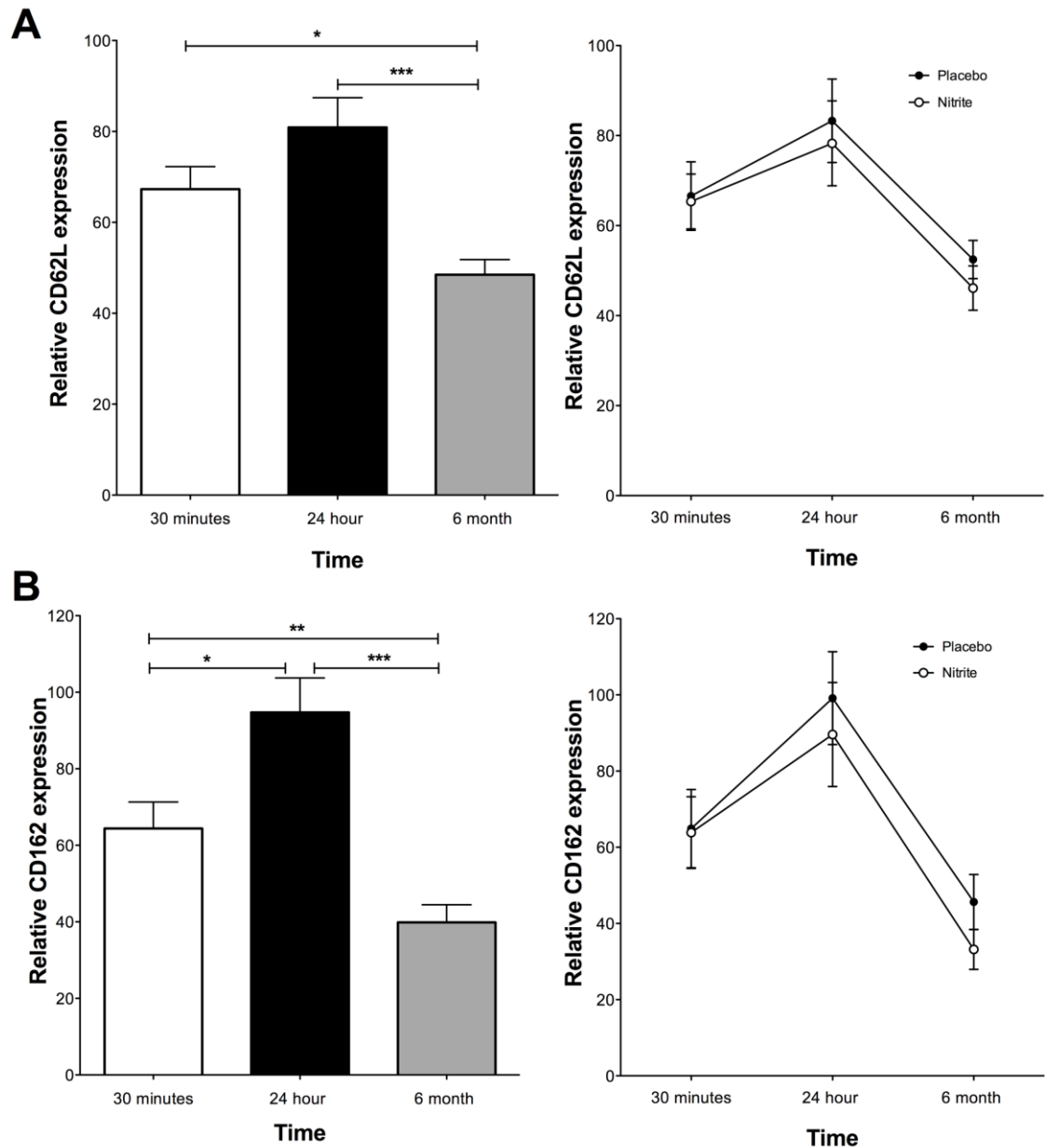
Differential leukocyte count was measured at baseline, 4 hours, 24 hours and 6 months after coronary reperfusion. Neutrophil cell count over time in all patients is shown in panel A. Panel B shows neutrophil cell count over time comparing nitrite (n=40) treated versus placebo (n=40). Panel C shows lymphocyte cell count assessed over time in all patients. Panel D shows lymphocyte count over time comparing nitrite treated versus placebo. Panel E shows monocyte cell count assessed over time in all patients with Panel F showing monocyte cell count over time comparing nitrite treated versus placebo. Data expressed as mean  $\pm$  SEM. \*\*\* $P < 0.0001$  using one-way ANOVA, with Bonferroni's multiple comparison test. ##  $P < 0.01$ , for two-way repeated measures ANOVA, \* $P < 0.05$  for two-way repeated measures ANOVA with Bonferroni's multiple comparison test.



**Figure 4.10 Neutrophil Cd11b Relative Fluorescence Intensity.**

Relative Fluorescence Intensity (RFI) for CD11b was measured at 30 minutes, 24 hours and 6 months after coronary reperfusion. RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel A. Panel B shows RFI over time of nitrite (21 patients) treated versus placebo (23 patients). Data expressed as mean  $\pm$  SEM. \*\*\* $P < 0.0001$  using 1-way ANOVA, with Bonferroni's multiple comparison test. ##= $P < 0.01$ , for two-way repeated measures ANOVA. \*\* for  $p < 0.001$  using two-repeated measures ANOVA with Bonferroni's multiple comparison test.

Neutrophil CD62L RFI changed substantially over time, with levels increasing from 30 minutes to 24 hours post reperfusion before declining significantly to lowest levels at 6 months (Figure 4.11A). This pattern was duplicated in CD162 RFI with increased expression at 24 hours and lowest levels at 6 months (Figure 4.11C). No difference was seen in either neutrophil CD162 or CD62L RFI between the nitrite or placebo groups over the time-points (Figure 4.11B and D).



**Figure 4.11 Neutrophil CD62L/162 Relative Fluorescence Intensity.**

Relative Fluorescence Intensity (RFI) for CD62L/CD162 was measured at 30 minutes, 24 hours and 6 months after coronary reperfusion. CD62L RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel A. Panel B shows CD62L RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD162 RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel C. Panel D shows CD162 RFI over time of nitrite (21 patients) treated versus placebo (23 patients). Data expressed as mean  $\pm$  SEM. \*\*\* $P < 0.0001$  using 1-way ANOVA, with Bonferroni's multiple comparison test. Panel B and D comparisons performed using two-way repeated measures ANOVA.

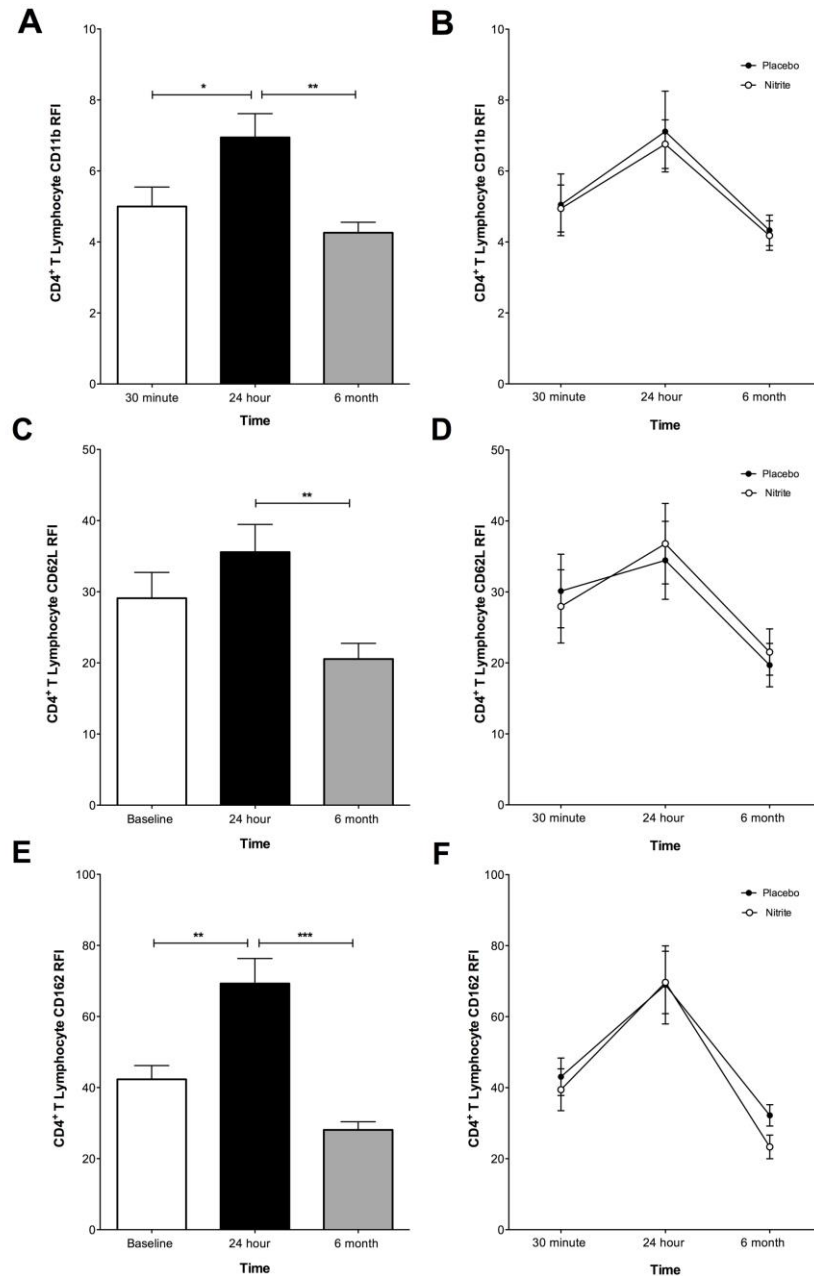
#### **4.3.2.5 T Lymphocytes**

##### **4.3.2.5.1 CD4<sup>+</sup> T lymphocytes**

CD4<sup>+</sup> T lymphocyte CD11b, CD62L and CD162 expression all increased from 30 minutes to 24 hours post reperfusion with significantly lower levels seen at 6 months after reperfusion (Figure 4.12A,C and E). There were comparable levels of CD11b expression in the nitrite treated patients compared to placebo over the time-points ( $p=0.745$ , figure 4.12B). This pattern was duplicated in the relative expression of CD62L and CD162 with no difference seen in the expression of either CD162 ( $p=0.487$ ) or CD62L ( $p=0.864$ ) between the nitrite or placebo groups over the time-points (Figure 4.12D, F).

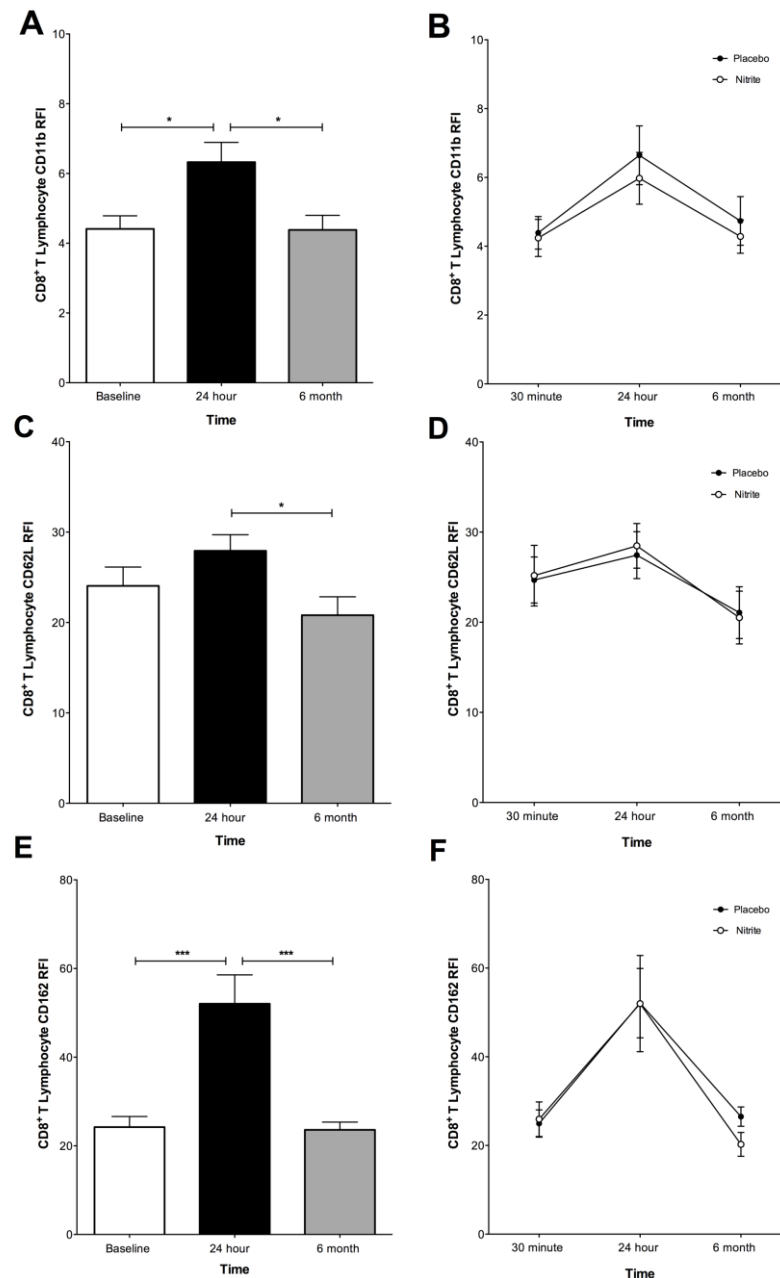
##### **4.3.2.5.2 CD8<sup>+</sup> T lymphocytes**

CD8<sup>+</sup> T lymphocyte CD11b and CD162 expression all increased from 30 minutes to 24 hours post reperfusion with significantly lower levels seen at 6 months after reperfusion (Figure 4.13A and E). CD62L levels increased marginally from 30 minute to 24 hours with a significant decrease by 6 months (Figure 4.13C). There was slightly lower levels of CD11b expression in the nitrite treated patients compared to placebo over the time-points but this was not significant ( $P=0.435$ , figure 4.13B). There was no difference seen in the relative expression of either CD162 ( $p=0.717$ ) or CD62L ( $p=0.889$ ) between the nitrite and placebo groups over the time-points (Figure 4.13D, F).



**Figure 4.12 CD4<sup>+</sup> T Lymphocyte CD11b/CD62L/162 Relative Fluorescence Intensity.**

Relative Fluorescence Intensity (RFI) for CD11b/CD62L/CD162 was measured at 30 minutes, 24 hours and 6 months after coronary reperfusion. CD11b RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel A. Panel B shows CD11b RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD62L RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel C. Panel D shows CD62L RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD162 RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel E. Panel F shows CD162 RFI over time of nitrite (21 patients) treated versus placebo (23 patients). Data expressed as mean ± SEM. \*\*P<0.01, \*P<0.05 using 1-way ANOVA, with Bonferroni's multiple comparison test. Panel B, D, F comparisons performed using two-way repeated measures ANOVA.



**Figure 4.13 CD8<sup>+</sup> T lymphocyte Relative Fluorescence Intensity.**

Relative Fluorescence Intensity (RFI) for CD11b/CD62L/CD162 was measured at 30 minutes, 24 hours and 6 months after coronary reperfusion. CD11b RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel A. Panel B shows CD11b RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD62L RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel C. Panel D shows CD62L RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD162 RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel E. Panel F shows CD162 RFI over time of nitrite (21 patients) treated versus placebo (23 patients). Data expressed as mean ± SEM. \*\*\*P<0.0001, \*P<0.05 using 1-way ANOVA, with Bonferroni's multiple comparison test. Panel B, D, F comparisons performed using two-way repeated measures ANOVA.

#### **4.3.2.6 Monocytes**

Monocytes were split into inflammatory (classical) monocytes (CD14+/CD16-), intermediate monocytes (CD14+/CD16+) and non-classical/resident monocytes (CD14+/CD16++) (Passlick et al., 1989; Ziegler-Heitbrock et al., 2010; Rogacev et al., 2012)

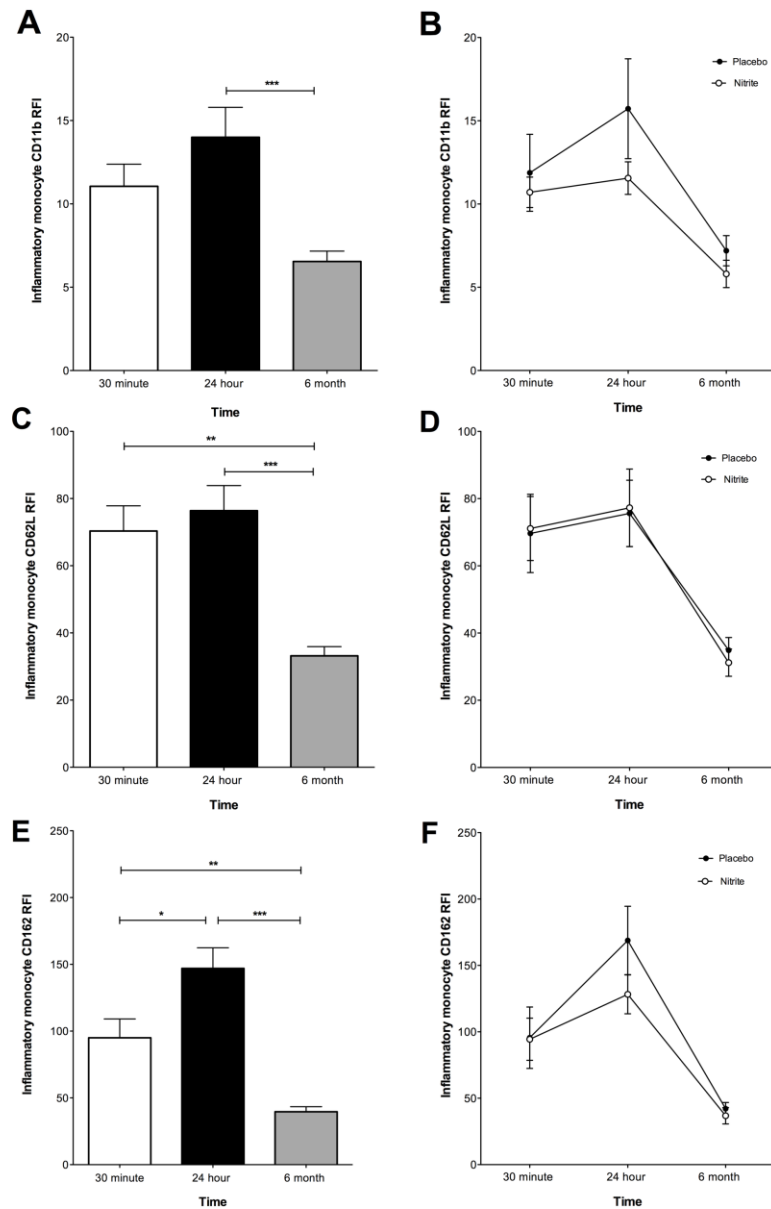
##### **4.3.2.6.1 Inflammatory monocytes (CD14+/CD16-)**

Inflammatory monocyte relative CD11b, CD62l and CD162 expression all increased from 30 minutes to 24 hours post reperfusion however only CD162 increased significantly. There were significantly lower levels of each activation marker seen at 6 months after reperfusion (Figure 4.14 A, C and E). There was a trend to lower levels of CD11b expression in the nitrite treated patients compared to placebo over the time-points with the largest difference at 24 hours however this did not reach statistical significance (p=0.120, figure 4.14B). There was no difference in the relative expression of either CD162 (p=0.274) or CD62L (p=0.981) between the nitrite or placebo groups over the time-points (Figure 4.14D, F).

##### **4.3.2.6.2 Intermediate monocytes (CD14+/CD16+)**

Intermediate monocyte relative CD11b, CD62l and CD162 expression all increased from 30 minutes to 24 hours post reperfusion, however similar to the inflammatory monocytes only CD162 increased significantly. There were significantly lower levels of each activation marker seen at 6 months after





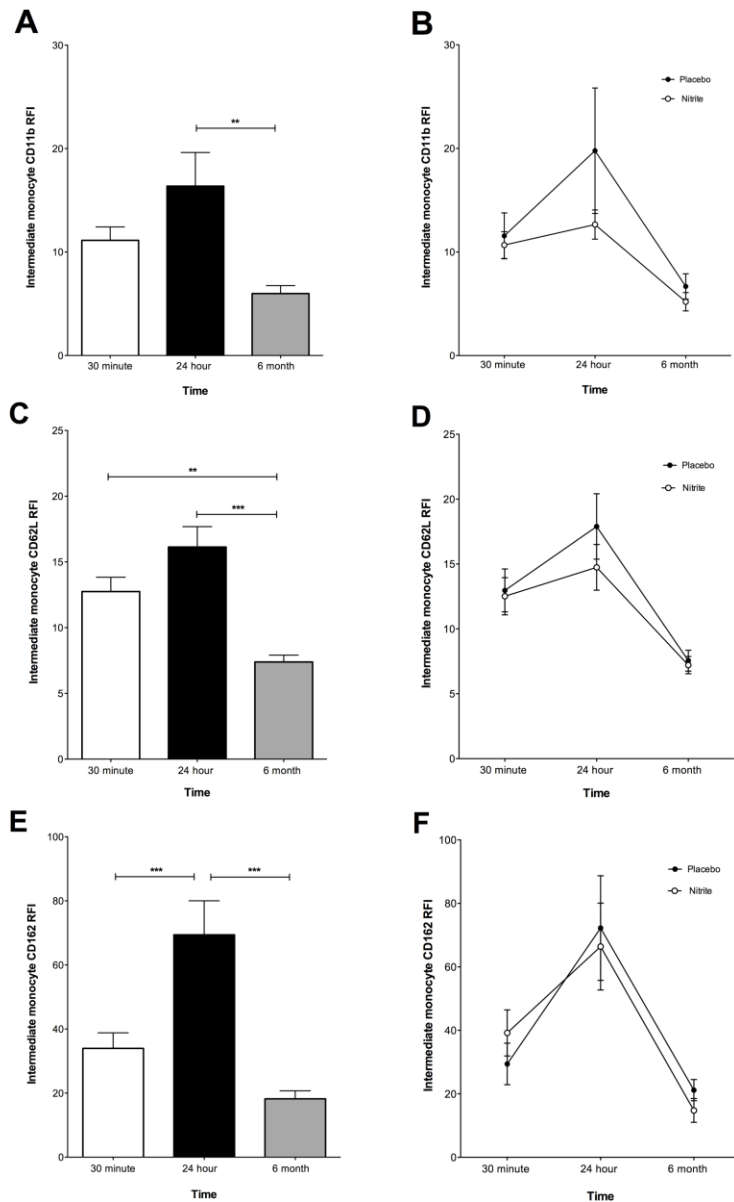
**Figure 4.14 Inflammatory monocyte Relative Fluorescence Intensity.**

Relative Fluorescence Intensity (RFI) for CD11b/CD62L/CD162 was measured at 30 minutes, 24 hours and 6 months after coronary reperfusion. CD11b RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel A. Panel B shows CD11b RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD62L RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel C. Panel D shows CD62L RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD162 RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel E. Panel F shows CD162 RFI over time of nitrite (21 patients) treated versus placebo (23 patients). Data expressed as mean  $\pm$  SEM. \*\*\* $P < 0.0001$ , \*\* $P < 0.01$ , \* $P < 0.05$  using 1-way ANOVA, with Bonferroni's multiple comparison test. Panel B, D, F comparisons performed using two-way repeated measures ANOVA

reperfusion (Figure 4.15 A, C and E). There was a trend to lower levels of CD11b expression in the nitrite treated patients compared to placebo over the time-points with the greatest difference between the two groups occurring at 24 hours however this did not reach statistical significance ( $p=0.190$ , figure 4.15B). There was no difference in the relative expression of either CD162 ( $p=0.920$ ) or CD62L ( $p=0.320$ ) between the nitrite or placebo groups over the time-points (Figure 4.15D, F).

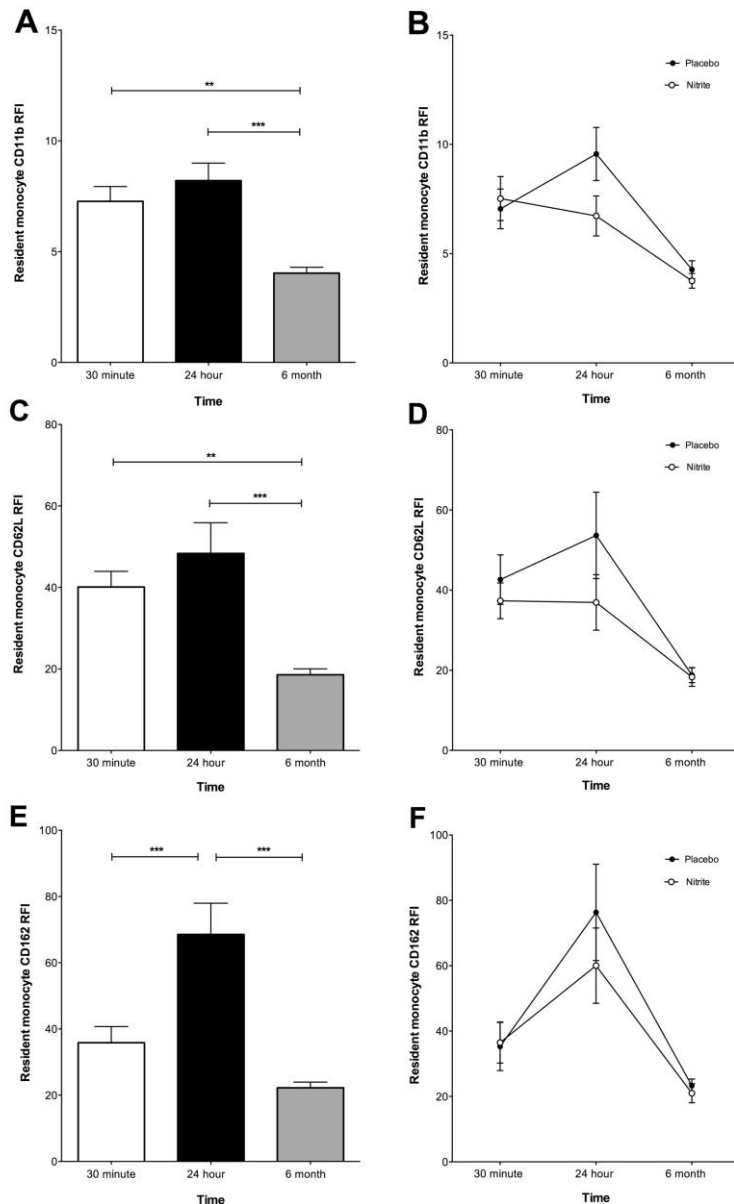
#### **4.3.2.6.3 Resident monocytes (CD14+/CD16++)**

Resident monocyte relative CD11b, CD62l and CD162 expression all increased from 30 minutes to 24 hours post reperfusion, however similar to both inflammatory and intermediary monocytes only CD162 increased significantly. There were significantly lower levels of each activation marker seen at 6 months after reperfusion (Figure 4.16 A, C and E). There was a trend to lower levels of CD11b expression in the nitrite treated patients compared to placebo over the time-points with the largest difference at 24 hours however this did not reach statistical significance ( $p=0.180$ , figure 4.16B). A similar pattern was seen in the relative expression of CD62L ( $p=0.144$ ) over time between the nitrite and placebo treated patients, with no difference in the relative expression of CD162L ( $p=0.320$ ) between the nitrite or placebo groups over the time-points (Figure 4.16D, F).



**Figure 4.15 Intermediate monocyte Relative Fluorescence Intensity.**

Relative Fluorescence Intensity (RFI) for CD11b/CD62L/CD162 was measured at 30 minutes, 24 hours and 6 months after coronary reperfusion. CD11b RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel A. Panel B shows CD11b RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD62L RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel C. Panel D shows CD62L RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD162 RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel E. Panel F shows CD162 RFI over time of nitrite (21 patients) treated versus placebo (23 patients). Data expressed as mean  $\pm$  SEM. \*\*\* $P < 0.0001$ , \*\* $P < 0.01$ , \* $P < 0.05$  using 1-way ANOVA, with Bonferroni's multiple comparison test. Panel B, D, F comparisons performed using two-way repeated measures ANOVA.



**Figure 4.16 Resident monocyte Relative Fluorescence Intensity.**

Relative Fluorescence Intensity (RFI) for CD11b/CD62L/CD162 was measured at 30 minutes, 24 hours and 6 months after coronary reperfusion. CD11b RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel A. Panel B shows CD11b RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD62L RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel C. Panel D shows CD62L RFI over time of nitrite (21 patients) treated versus placebo (23 patients). CD162 RFI assessed over time in 44 patients (23 placebo patients and 21 nitrite patients) is shown in panel E. Panel F shows CD162 RFI over time of nitrite (21 patients) treated versus placebo (23 patients). Data expressed as mean  $\pm$  SEM. \*\*\* $P < 0.0001$ , \*\* $P < 0.01$ , \* $P < 0.05$  using 1-way ANOVA, with Bonferroni's multiple comparison test. Panel B, D, F comparisons performed using two-way repeated measures ANOVA.

### **4.3.3 Inflammatory mediators**

Plasma levels of high-sensitivity C-reactive protein and monocyte-chemoattractant protein-1 were measured at baseline, 4 hours, 24 hours and 6 months post reperfusion.

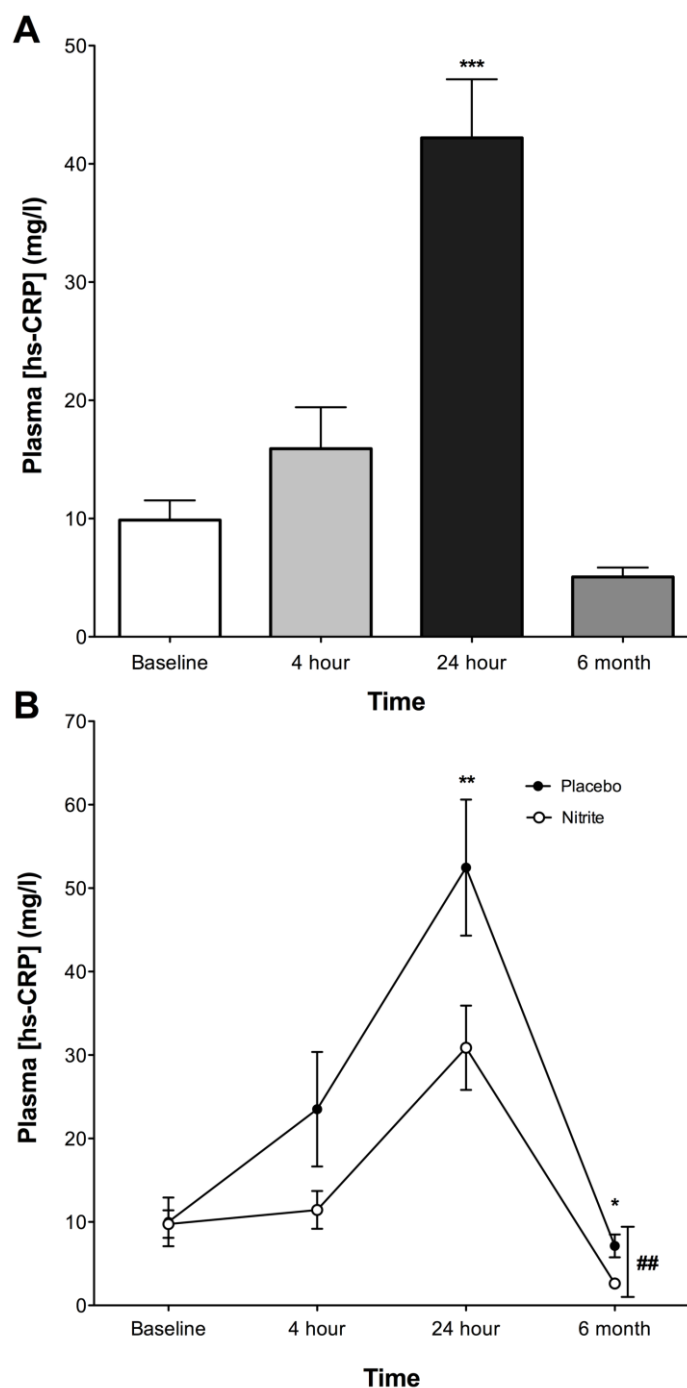
#### **4.3.3.1 High-sensitivity C-reactive protein**

Plasma hs-CRP increased from baseline at 4 hours, and then to its highest level by 24 hours. However, at 6 months levels had returned to below baseline levels (Figure 4.17A,  $p < 0.0001$ ). Between the treatment cohorts, plasma hs-CRP levels were comparable at baseline however in the nitrite treated patients the levels of hs-CRP over each of the remaining time-points were substantially reduced compared to placebo ( $p = 0.003$ ) with the greatest difference evident at 24 hours (Figure 4.17B). To determine whether the rise in hs-CRP was related to the size of the infarct post-hoc analyses were conducted using the levels of hs-CRP at their peak i.e 24 hours. These analyses demonstrated that plasma levels of hs-CRP are positively associated with infarct size ( $r = 0.337$ ,  $p = 0.005$ ) (Figure 4.19A) as assessed by cardiac enzymes.

#### **4.3.3.2 Monocyte-chemoattractant protein-1 (MCP-1)**

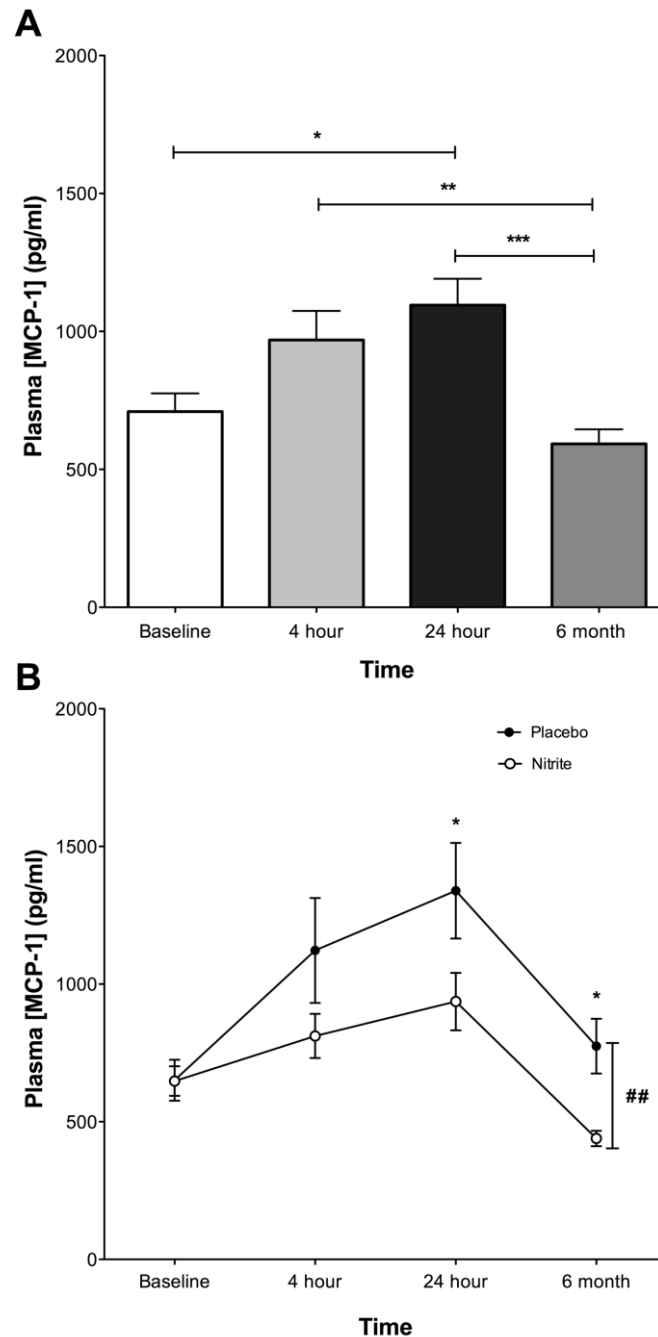
Plasma MCP-1 levels of the combined cohort increased from baseline at 4 hours and then to its highest level measured at 24 hours. However at 6 months levels had returned to below baseline values (Figure 4.18A,  $p < 0.0001$ ). Between the

two treatment cohorts plasma MCP-1 levels were comparable at baseline however in the nitrite treated patients the plasma levels of MCP-1 over the remaining time-points were substantially reduced compared to placebo ( $p=0.0058$ ) with the greatest difference evident at 24 hours (Figure 4.18B). Further post-hoc analyses demonstrated that levels of plasma MCP-1 are positively associated with infarct size ( $r=0.309$ ,  $p=0.020$ ) (Figure 4.19B) and levels of plasma hs-CRP ( $r=0.341$ ,  $p=0.005$ ) (Figure 4.19C).



**Figure 4.17 Effect of time and treatment on plasma hs-CRP.**

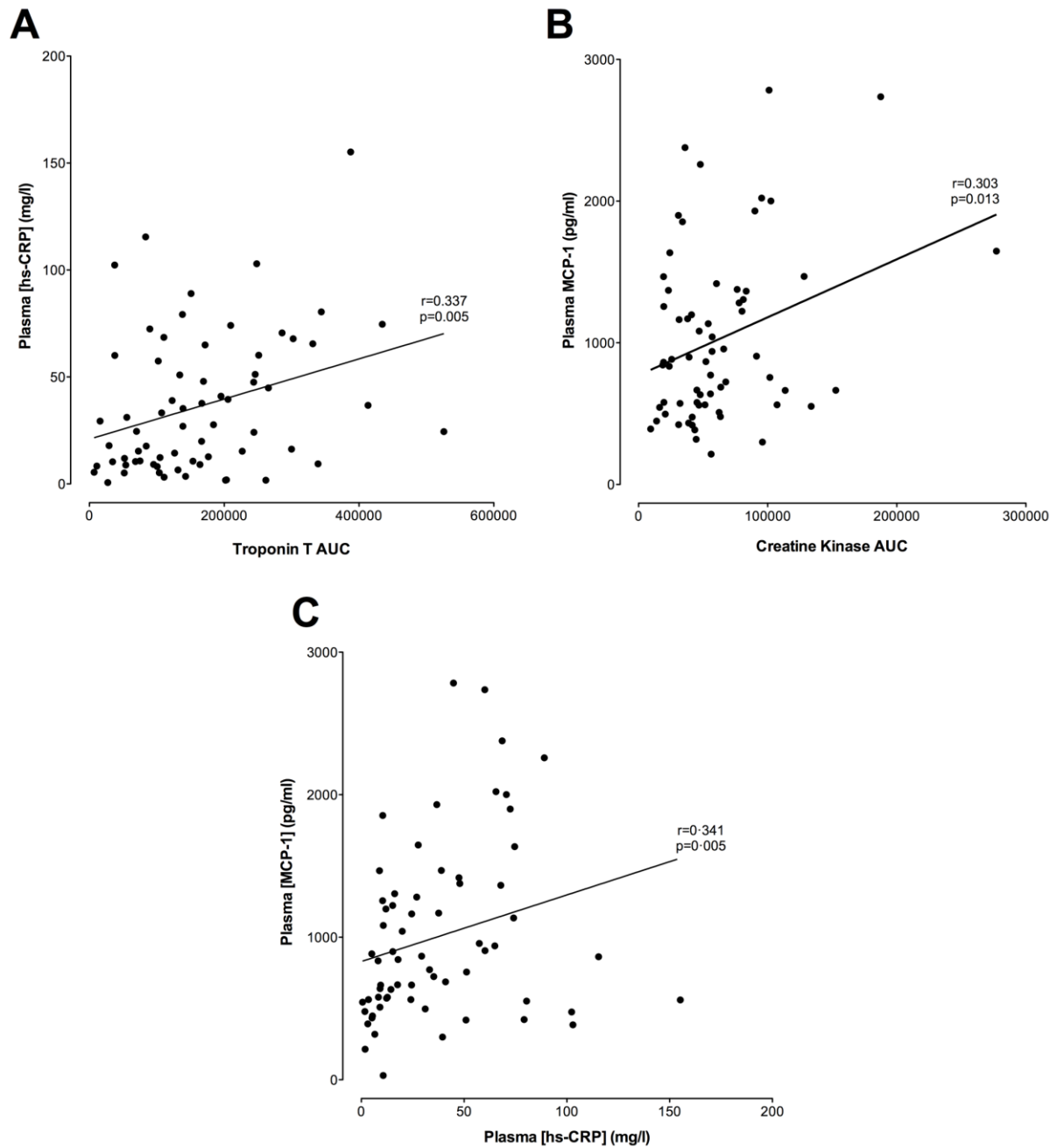
High-sensitivity C-reactive protein (hs-CRP) was measured at baseline, 4 hours, 24 hours and 6 months after coronary reperfusion. hs-CRP assessed over time in 79 patients shown in panel A. Panel B shows hs-CRP over time showing nitrite (39 patients) treated versus placebo (40 patients). Data expressed as mean  $\pm$  SEM. \*\*\* $P < 0.0001$  using 1-way ANOVA, with Bonferroni's multiple comparison test. ##= $P < 0.01$  using two-way repeated measures ANOVA, \*\*= $P < 0.01$ , \*= $P < 0.05$  using two-way repeated measures ANOVA with Bonferroni's multiple comparison test (Hs-CRP= High-sensitivity C-reactive protein).



**Figure 4.18 Effect of time and treatment on plasma MCP-1.**

Monocyte chemoattractant Protein-1 (MCP-1) measured at baseline, 4 hours, 24 hours and 6 months after coronary reperfusion. MCP-1 assessed over time in 74 patients (37 nitrite and 37 placebo) is shown in panel A. Panel B shows MCP-1 over time showing nitrite treated versus placebo. Data expressed as meanSEM. \*\*\* $P < 0.0001$ , \*\* $P < 0.01$ , \* $P < 0.05$  using 1-way ANOVA, with Bonferroni's multiple comparison test.. ##= $P < 0.01$  using two-way repeated measures ANOVA, \*= $P < 0.05$  using two-way repeated measures ANOVA with Bonferroni's multiple comparison test (Hs-CRP= High-sensitivity C-reactive protein). (MCP-1=monocyte chemoattractant protein 1)





**Figure 4.19 Associations between Hs-CRP, MCP-1 and infarct size.**

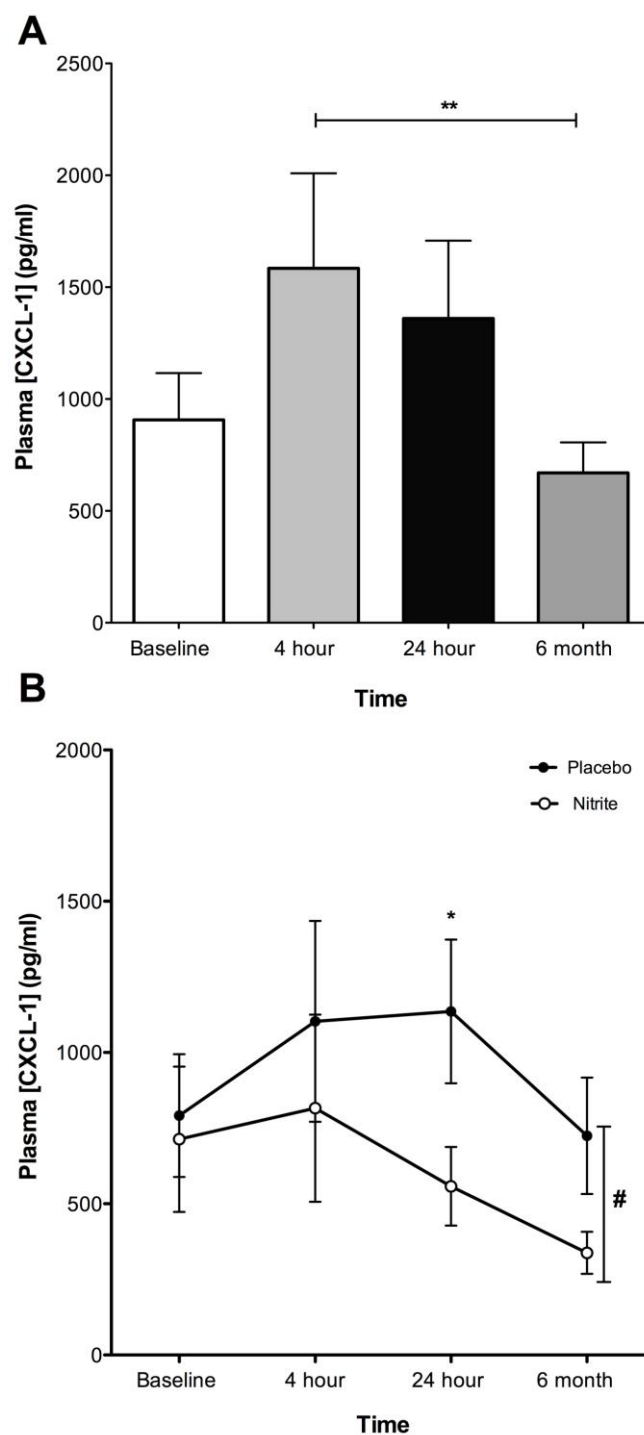
There was a significant positive association between levels of Hs-CRP measured at 24 hours and infarct size assessed by troponin T AUC as shown in panel A. Panel B depicts a similar positive association between MCP-1 levels at 24 hours and CK AUC. Panel C shows a positive correlation between MCP-1 and Hs-CRP levels measured at 24 hours. Correlations determined using Pearson's correlation coefficient. (MCP-1=monocyte chemoattractant protein 1, hs-CRP= High-sensitivity C-reactive protein, CK= Creatine Kinase, AUC=Area under the curve).

#### **4.3.3.3 Neutrophil chemokines**

Since the greatest changes were found in neutrophil numbers and activation states between treatment arms, the possibility that this might be related to differences in neutrophil chemokine levels was also investigated. Levels of CXCL-1 and CXCL-5 were measured in all patients (40 nitrite and 40 placebo) at baseline, 4 hours, 24 hours and 6 months post reperfusion.

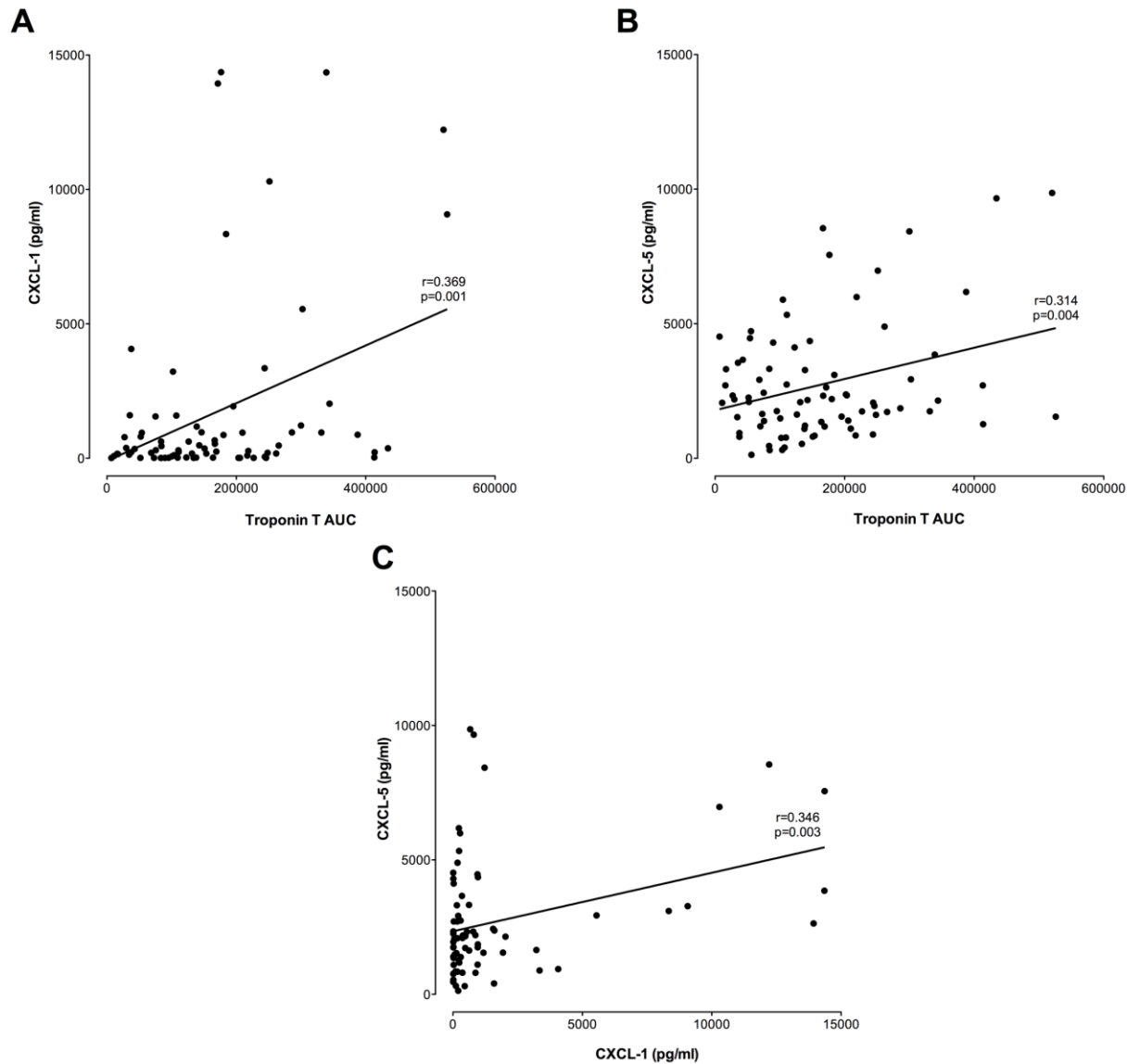
##### **4.3.3.3.1 CXCL-1**

Plasma CXCL-1 levels increased from baseline ( $907.27 \pm 208.83$  pg/ml) to the highest levels seen at 4 hours ( $1584.37 \pm 424.99$  pg/ml), with a slight decrease by 24 hours ( $1360.39 \pm 347.62$  pg/ml). However at 6 months levels had returned to below baseline values (Figure 4.20A). Between the two treatment cohorts plasma CXCL-1 levels were comparable at baseline however in the nitrite treated patients the plasma levels of CXCL-1 over the remaining time-points were substantially reduced compared to placebo ( $p=0.04$ ) with the greatest difference evident at 24 hours ( $p=0.043$ ) (Figure 4.20B). Further post-hoc analyses demonstrated that levels of plasma CXCL-1 are positively associated with infarct size ( $r=0.369$ ,  $p=0.001$ ) (Figure 4.21A).



**Figure 4.20 Effect of time and treatment on plasma CXCL-1.**

CXCL-1 levels were measured at baseline, 4 hours, 24 hours and 6 months after coronary reperfusion. CXCL-1 assessed over time in all patients is shown in panel A. Panel B shows CXCL-1 over time showing nitrite treated versus placebo groups. Data expressed as mean  $\pm$  SEM, \*\* $P < 0.01$  using 1-way ANOVA, with Bonferroni's multiple comparison test. #= $P < 0.05$  using two-way repeated measures ANOVA, \*= $P < 0.05$  using two-way repeated measures ANOVA with Bonferroni's multiple comparison test.

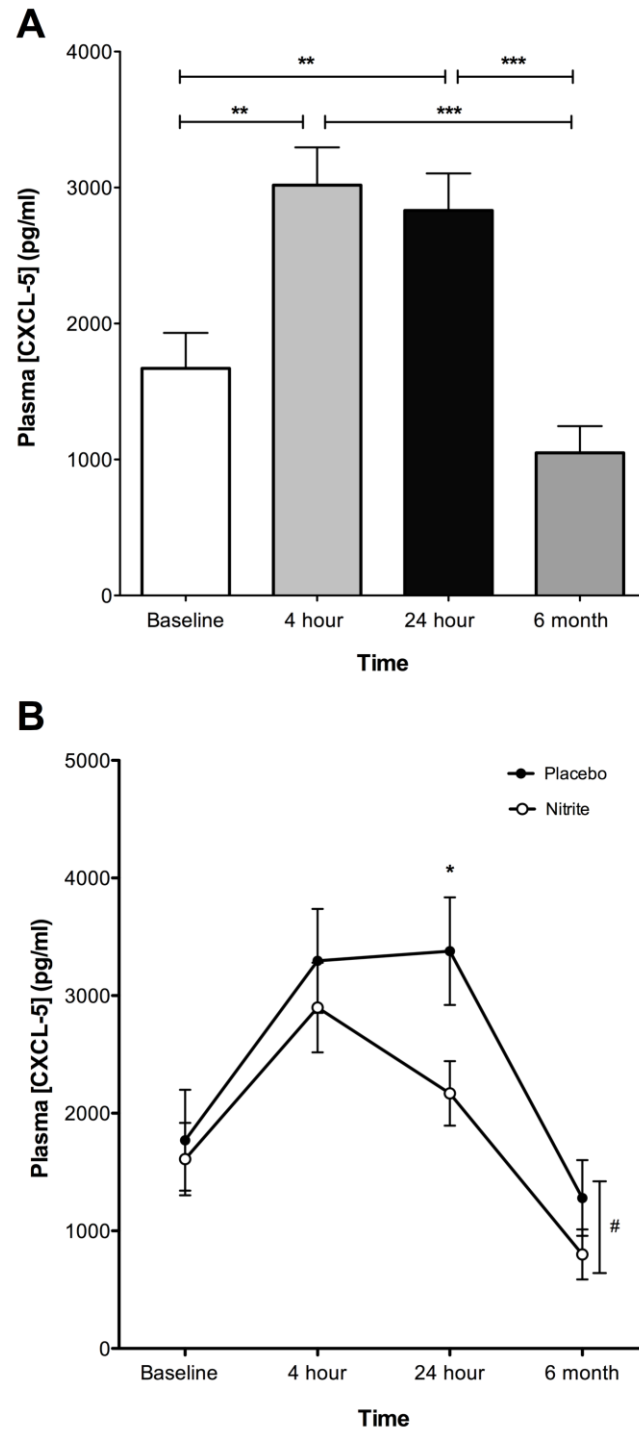


**Figure 4.21 Associations between CXCL-1, CXCL-5 and infarct size.**

There was a significant positive association between levels of CXCL-1 measured at 24 hours and infarct size assessed by troponin T AUC as shown in panel A. Panel B depicts a similar positive association between CXCL-5 levels at 24 hours and Troponin T AUC. Panel C shows a positive correlation between CXCL-1 and CXCL-5 levels measured at 24 hours. Correlations determined using Pearson's correlation coefficient.

#### 4.3.3.3.2 CXCL-5

Plasma CXCL-5 levels increased from baseline ( $1670.85 \pm 261.10$  pg/ml) to the highest levels seen at 4 hours ( $3018.14 \pm 277.76$  pg/ml), with a slight decrease by 24 hours ( $2831.83 \pm 272.17$  pg/ml). However at 6 months levels had returned to below baseline values (Figure 4.22A,  $P < 0.0001$ ). Between the two treatment cohorts plasma CXCL-5 levels were comparable at baseline however in the nitrite treated patients the plasma levels of CXCL-1 over the remaining time-points were substantially reduced compared to placebo ( $p = 0.04$ ) with the greatest difference evident at 24 hours ( $p = 0.044$ ) (Figure 4.22B). Further post-hoc analyses demonstrated that levels of plasma CXCL-5 are positively associated with infarct size ( $r = 0.314$ ,  $p = 0.004$ ) (Figure 4.21B) and levels of plasma CXCL-1 ( $r = 0.346$ ,  $p = 0.003$ ) (Figure 4.21C).



**Figure 4.22 Effect of time and treatment on plasma CXCL-5.**

CXCL-5 levels were measured at baseline, 4 hours, 24 hours and 6 months after coronary reperfusion. CXCL-5 assessed over time in all patients is shown in panel A. Panel B shows CXCL-5 over time showing nitrite treated versus placebo groups. Data expressed as mean  $\pm$  SEM. \*\*\* $P < 0.0001$ , \*\* $P < 0.01$  using 1-way ANOVA, with Bonferroni's multiple comparison test. #= $P < 0.05$  using two-way repeated measures ANOVA, \*=  $P < 0.05$  using two-way repeated measures ANOVA with Bonferroni's multiple comparison test.

#### **4.3.4 Plasma NOx**

##### **4.3.4.1 Acute measurement**

In chapter 3 plasma [NO<sub>2</sub><sup>-</sup>] measurements at 30 minutes demonstrated effective administration of the IMP. However plasma NOx levels are also a reflection of systemic inflammation as a consequence of iNOS expression and activity. In light of this levels of both nitrite and nitrate were increased in the whole cohort at each time-point measured. Plasma NOx levels were measured at baseline, 30 minutes, 4 hours, and 24 hours post reperfusion in all patients (40 patients in the placebo group and 40 patients in the nitrite group). Plasma levels of nitrite in the whole cohort significantly increased from baseline to 30 minutes post reperfusion with lower levels evident at both 4 hours and 24 hours (Figure 4.23A,  $P<0.0001$ ).

As indicated previously the rise in nitrite levels at 30 minutes post reperfusion is predominantly occurring within the nitrite treated patients ( $P<0.0001$ , figure 3.3A, table 4.1) however a rise was also evident in the placebo group although this did not reach statistical significance ( $P=0.22$ , Figure 3.3C, table 4.1). At 4 and 24 hours after reperfusion plasma nitrite levels were significantly lower in the nitrite treated patients compared to placebo ( $p=0.423$ , table 4.1)

Plasma levels of nitrate in the whole cohort decreased steadily over the time-period measurements with significantly lower levels evident at 24 hours compared to baseline (Figure 4.23B,  $P<0.05$ ). Baseline levels of nitrate were

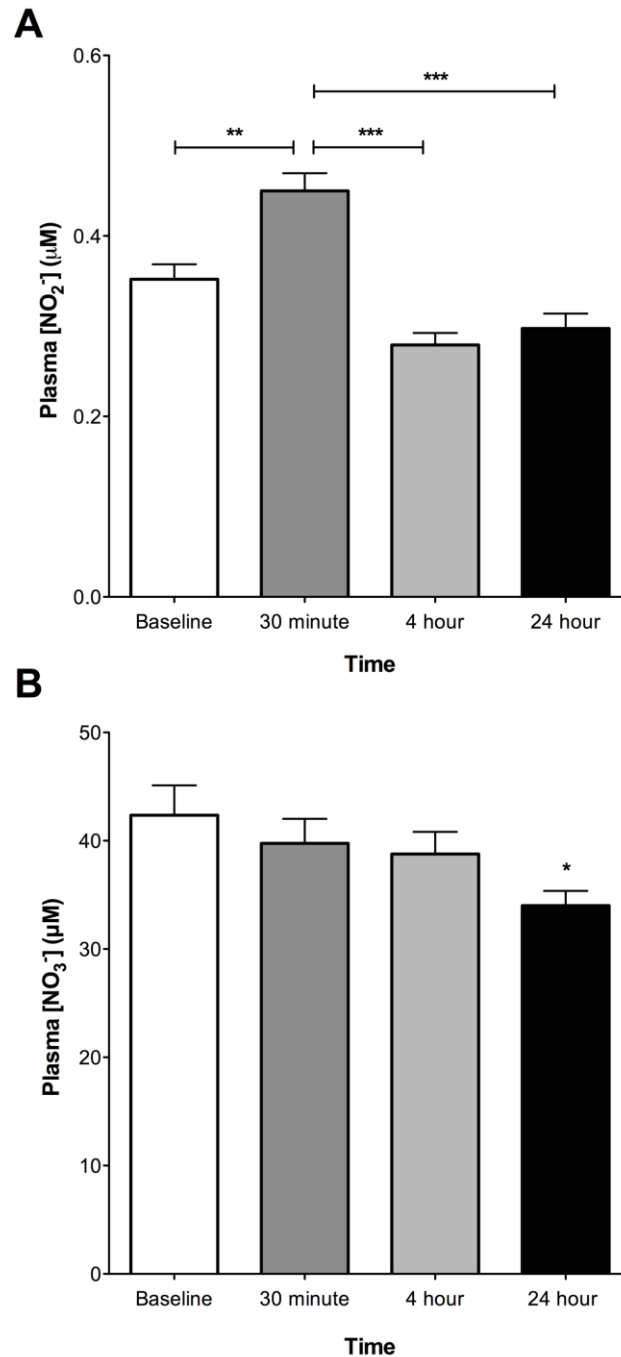
similar between the nitrite and placebo treated patients with neither treatment group having an elevation in nitrate levels at 30 minutes post reperfusion (Nitrite group  $p=0.788$ , placebo  $p=0.868$ , Figure 3.3B and D, Table 4.1). Levels of nitrate at 4 and 24 hours after reperfusion were higher in the nitrite treated patients compared to placebo, however this difference was not significant ( $p=0.10$ , Table 4.1)

	Baseline	30 minutes	4 hours	24 hours
<b>NO<sub>2</sub><sup>-</sup> (μM)</b>				
Nitrite	0.34 (0.30-0.38)	0.47 (0.41-0.53)	0.26 (0.24-0.29)*	0.27 (0.23-0.29)*
Placebo	0.37 (0.32-0.42)	0.40 (0.35-0.45)	0.30 (0.25-0.34)	0.32 (0.26-0.38)
<b>NO<sub>3</sub><sup>-</sup> (μM)</b>				
Nitrite	37.91 (33.45-42.38)	38.88 (33.88-43.88)	36.26 (31.67-40.86)	36.08 (32.45-39.72)
Placebo	40.44 (33.71-47.17)	40.62 (32.87-48.38)	32.88 (28.74-37.01)	32.14 (27.94-36.34)

**Table 4.2 Acute NO<sub>x</sub> measurements over time by treatment group**

Plasma NO<sub>x</sub> levels measured at baseline, 30 minutes, 4 hours, and 24 hours after coronary reperfusion in all patients (40 nitrite, 40 placebo). Data expressed as mean (95% CI). \* $P<0.05$  using two-way repeated measures ANOVA comparing nitrite versus placebo at 4 and 24 hours. (NO<sub>2</sub><sup>-</sup>=Nitrite, NO<sub>3</sub><sup>-</sup>=Nitrate)



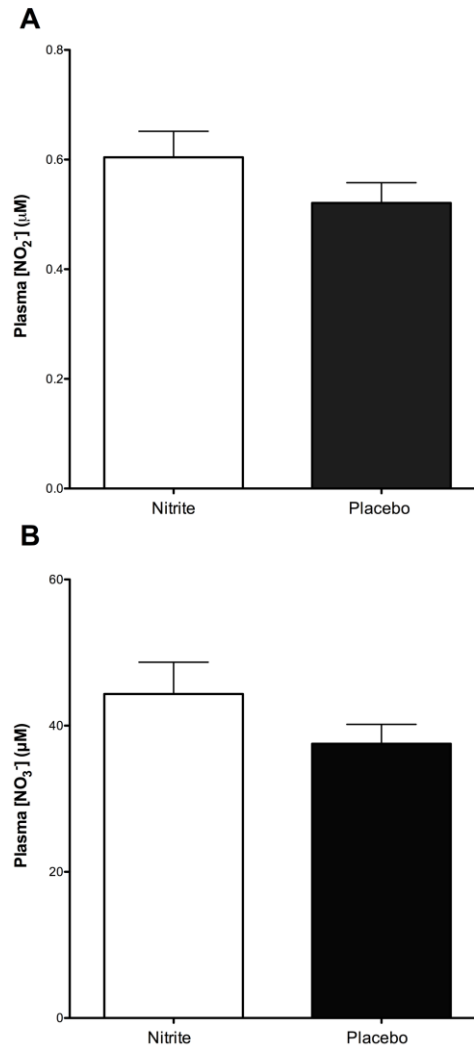


**Figure 4.23 Effect of time and treatment on plasma NO<sub>x</sub> levels.**

Plasma NO<sub>x</sub> levels were measured at baseline, 30 minutes, 4 hours, and 24 hours after coronary reperfusion. Plasma NO<sub>2</sub><sup>-</sup> assessed over time in all patients is shown in panel A. Panel B shows plasma NO<sub>3</sub><sup>-</sup> assessed over time in all patients. Data expressed as mean ± SEM. \*\*\*P<0.0001, \*\*P<0.01, \*P<0.05 using 1-way ANOVA, with Bonferroni's multiple comparison test. (NO<sub>2</sub><sup>-</sup> = Nitrite, NO<sub>3</sub><sup>-</sup> = Nitrate)

#### 4.3.4.2 6 month NOx levels

There was a trend to higher levels of plasma nitrite and nitrate at 6 months in the nitrite treated patients compared to placebo however these differences were not significant (Figure 4.24).



**Figure 4.24 Six month plasma NOx.**

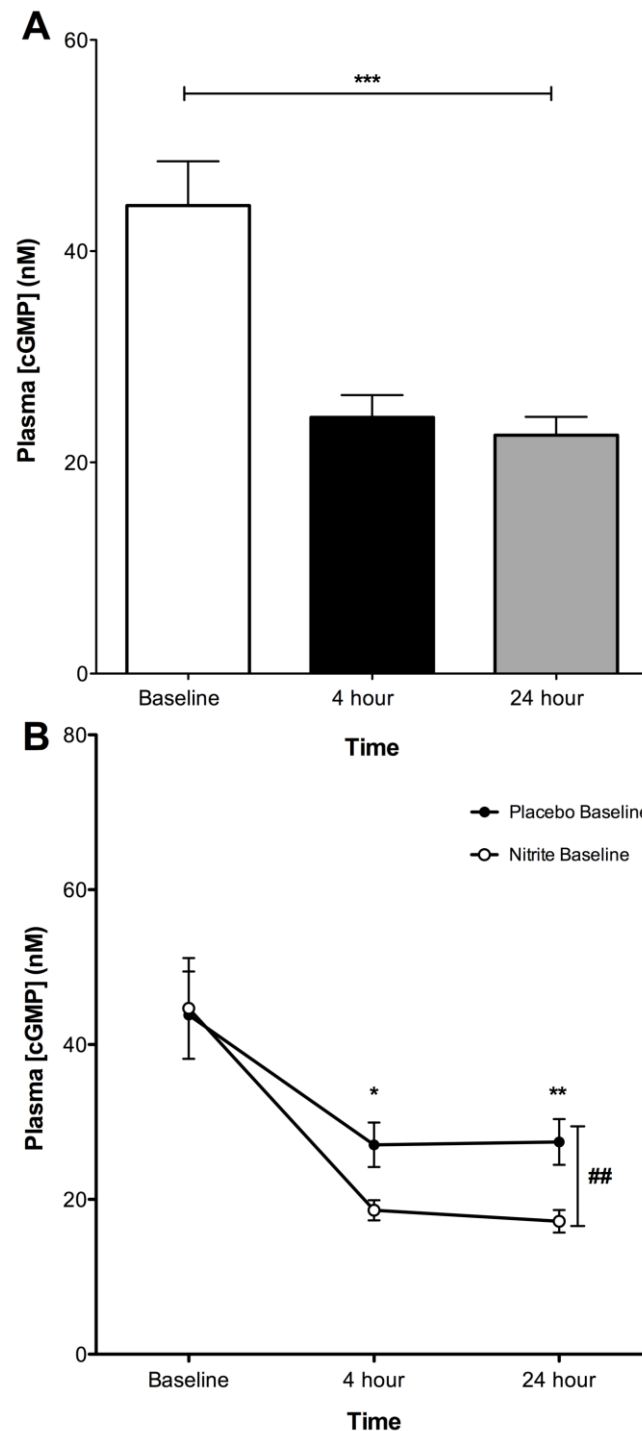
Plasma NOx levels measured at 6 months after coronary reperfusion in all patients. Panel A shows plasma NO<sub>2</sub><sup>-</sup> levels, and panel B shows NO<sub>3</sub><sup>-</sup> levels. Data expressed as mean ± SEM. Statistical comparisons between groups performed using unpaired T test. (NO<sub>2</sub><sup>-</sup> = Nitrite, NO<sub>3</sub><sup>-</sup> = Nitrite).

### **4.3.5 Plasma cGMP**

#### **4.3.5.1 Acute measurement**

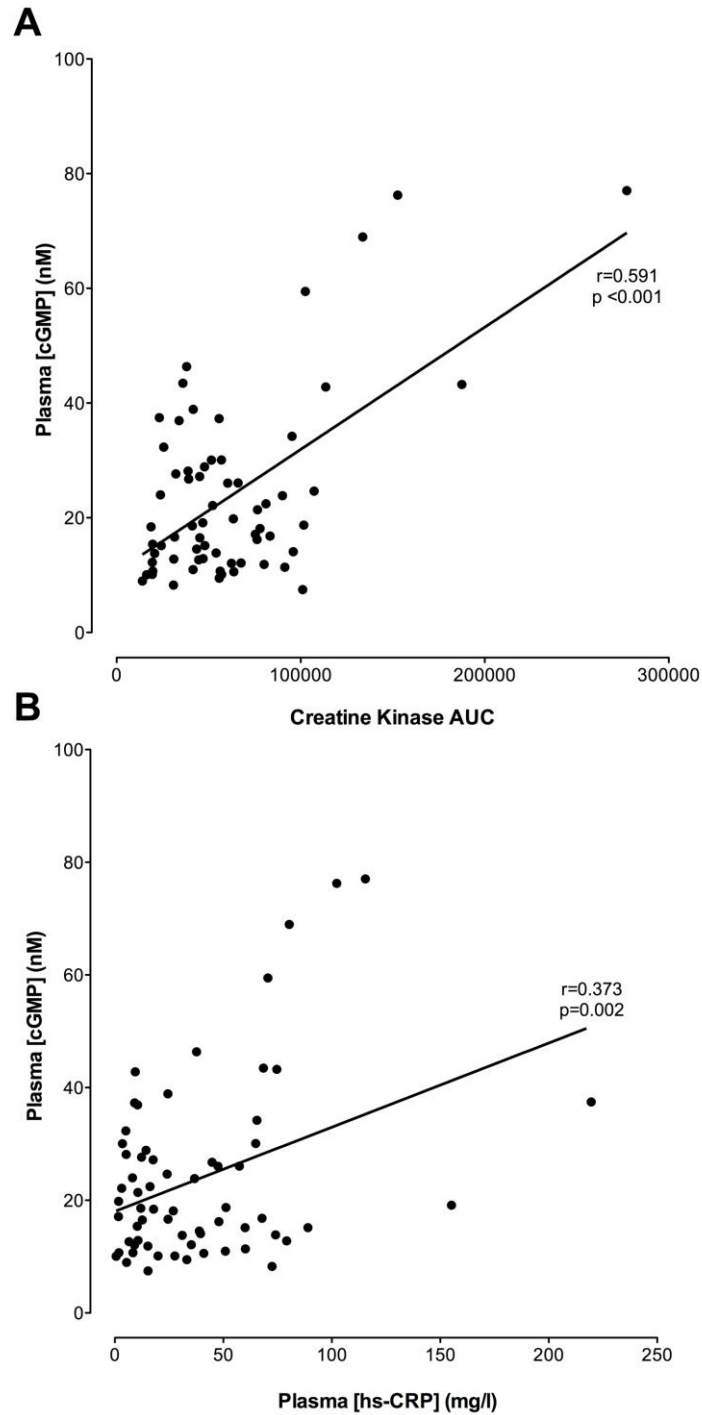
Levels of plasma cGMP were measured at baseline, 4 hours, and 24 hours post reperfusion. Levels of cGMP were highest at baseline and were significantly decreased at both 4 and 24 hours (Figure 4.25,  $P < 0.0001$ ).

Levels of cGMP were comparable at baseline between the nitrite and placebo treated patients but were significantly lower in the nitrite treated patients over the time-period ( $p = 0.0002$ ) with the greatest difference evident at 24 hours ( $20.45 \pm 2.60$  vs  $26.99 \pm 2.91$  nM) (Figure 4.24). Post-hoc analyses demonstrated that levels of cGMP at 24 hours are positively associated with both infarct size ( $r = 0.591$ ,  $p < 0.001$ ) and hs-CRP ( $r = 0.373$ ,  $p = 0.002$ ) (Figure 4.26).



**Figure 4.25 Effect of time and treatment on plasma cGMP levels.**

Levels of cGMP measured at baseline, 4 hours, and 24 hours after coronary reperfusion. C-GMP assessed over time in 76 patients shown in panel A. Panel B shows c-GMP over time showing nitrite (38 patients) treated versus placebo (38 patients). Data expressed as mean  $\pm$  SEM. \*\*\* $P < 0.0001$  using 1-way ANOVA, with Bonferroni's multiple comparison test, ##= $P < 0.001$  using two-way repeated measures ANOVA, \*=  $P < 0.05$ , \*\*= $P < 0.01$  using two-way repeated measures ANOVA with Bonferroni's multiple comparison test.

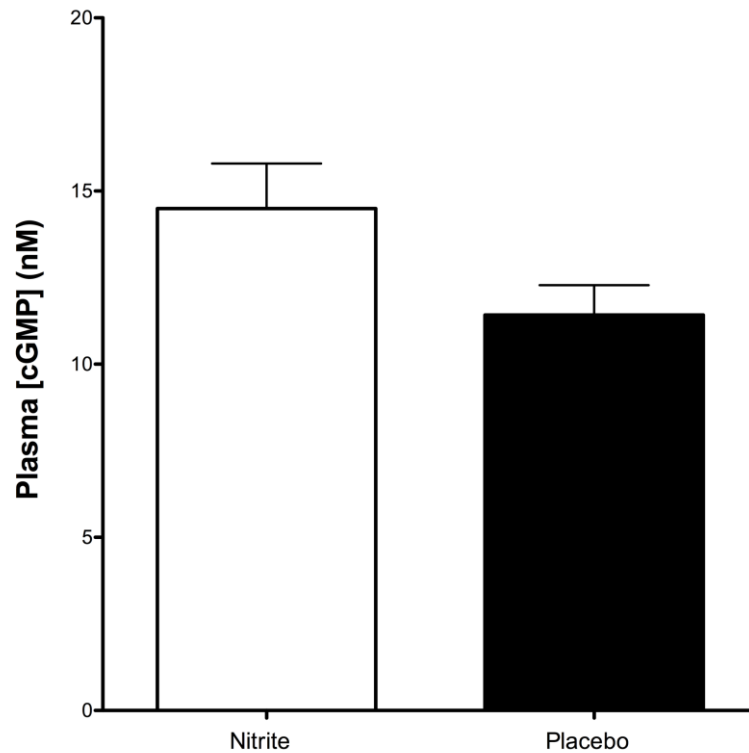


**Figure 4.26 Associations between cGMP levels, inflammation and infarct size.**

There was a significant positive association between levels of cGMP measured at 24 hours and infarct size assessed by Creatine Kinase AUC in the TIMI  $\leq 1$  subgroup shown in panel A. Panel B shows a significant positive association between levels of cGMP measured at 24 hours and plasma hs-CRP measured at 24 hours in the TIMI  $\leq 1$  subgroup. Correlations were determined using Pearson's correlation coefficient.

#### 4.3.5.2 6 month cGMP levels

At 6 months there was a trend to higher plasma levels of cGMP in the nitrite group compared to placebo but this did not reach statistical significance ( $p=0.052$ , Figure 4.27)



**Figure 4.27 Six-month plasma cGMP.**

Plasma cGMP levels measured at 6 months after coronary reperfusion in 75 patients (37 nitrite and 38 placebo). Data expressed as mean  $\pm$  SEM. Statistical comparisons between groups performed using unpaired T test. (cGMP = cyclic guanosine monophosphate).

#### 4.4 Summary

1. In patients following primary PCI for STEMI platelet reactivity was increased with elevated P-selectin expression that then decreased over time. The level of activation was significantly decreased in the nitrite compared to the placebo group.
2. Despite anti-thrombotic therapy (anti-platelets, heparin and GPIIb/IIIa inhibition) ex-vivo exposure of platelets to activating stimuli, ADP and collagen resulted in increased P-selectin expression and platelet aggregation. These responses decreased over time in the whole cohort but the decreases were substantially greater in the nitrite treated patients.
3. The decrease in platelet reactivity following nitrite was maintained out to 6 months after reperfusion and both P-selectin expression and the platelet aggregatory response were positively associated with myocardial infarct size.
4. In the whole cohort total circulating leukocytes were raised post reperfusion and then decreased over time. This rise was driven by increases in neutrophil numbers.
5. In nitrite treated patients the levels of neutrophils were suppressed compared to placebo and this was specifically associated with a decrease in the level of activation markers involved in adhesion namely CD11b.
6. There were no significant differences in the number of other leukocyte populations (monocytes and lymphocytes) or activation markers expressed by these cells between the treatment groups.
7. In the whole cohort the levels of inflammatory mediators were increased

post-reperfusion, this included hs-CRP and both neutrophil and monocyte chemokines.

8. The plasma levels of hs-CRP, neutrophil chemokines (CXCL-1 and CXCL-5) and MCP-1 were significantly lower in the nitrite group compared to placebo with the greatest difference evident at 24 hours.
9. Levels of MCP-1, Hs-CRP and the neutrophil chemokines (CXCL-1 and CXCL-5) were positively associated with infarct size
10. Whilst acutely nitrite levels were elevated at 30 minutes post-reperfusion this effect was largely due to the intervention in the nitrite arm.
11. In general over time both nitrite and nitrate levels decreased post reperfusion. This effect was greater in the nitrite treated patients.



## **Chapter 5.0**

# **The investigation of the effect of nitrite on endothelial and smooth muscle cell growth and viability**

## 5.1 Introduction

In all patients undergoing PCI for the treatment of AMI endovascular stents are placed following the angioplasty procedure to prevent the vessel from collapsing or re-occluding again. A major limitation of this technique is re-growth of the tissue within the stented part of the artery, known as in-stent restenosis (ISR). The introduction of drug-eluting stents (DESs) was seen as a solution to this problem and have reduced the incidence of ISR considerably (Regar et al., 2002), however attention has shifted to the safety of these devices because of evidence suggesting an association with late-stent thrombosis (Stolker et al., 2010) (a process associated with inhibition of/delayed re-endothelialisation). Therefore there have been calls for therapies that prevent restenosis and thrombosis, to improve the safety and efficacy of coronary stenting (Jukema et al., 2012).

Evidence exists supporting the concept that NO supplementation may be a viable solution to the above phenomena. As mentioned in chapter 1, section 13, NO plays a pivotal role as a multi-functional anti-platelet, anti-proliferative and vasorelaxant mediator of homeostasis that counteracts the restenotic process. Deficiencies in NO bioavailability have been shown to contribute to occlusive vascular diseases (Boger et al., 1996; Boger et al., 1997) and indeed genetic eNOS variants are also associated with clinical outcomes following PCI (Gorchakova et al., 2003). Additionally extensive pre-clinical studies across several species have demonstrated that NO supplementation in the form of NO donors inhibit smooth muscle cell proliferation (mediated via elevations of the cell cycling inhibitor p21<sup>waf1/cip1</sup>) in vitro (Ishida et al., 1997; Bauer et al., 2001; Alef et al., 2011) and

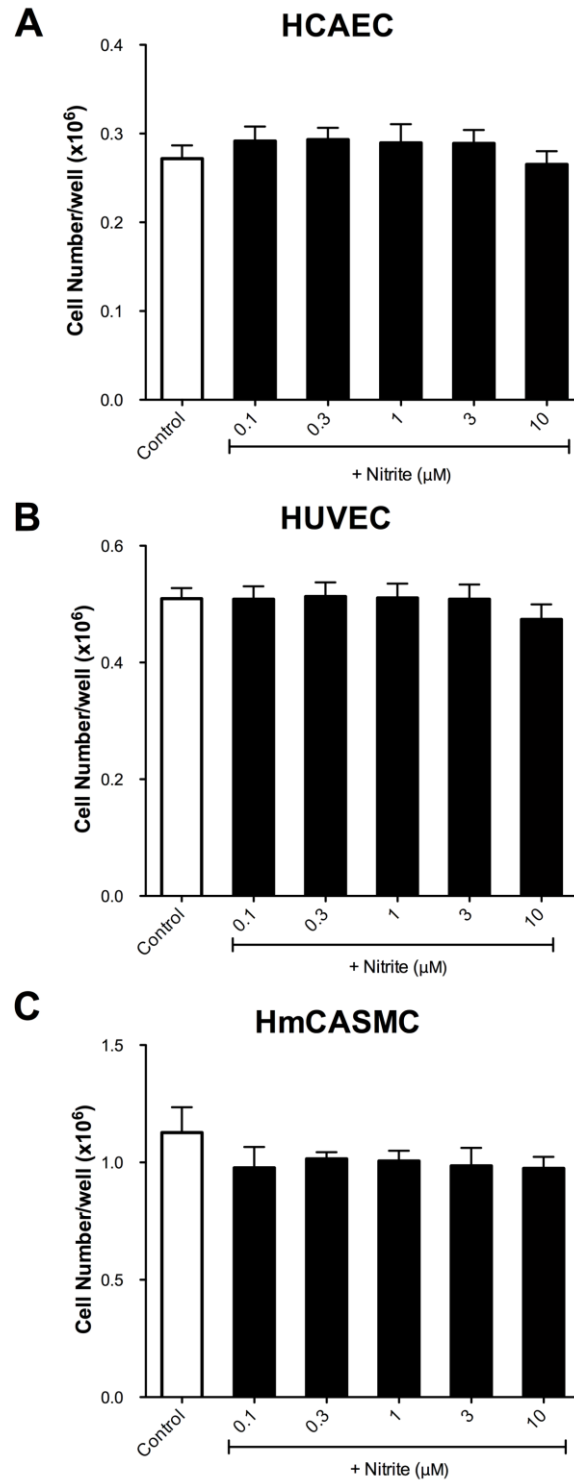
subsequent balloon induced experimental stenosis of coronary arteries (Janero and Ewing, 2000). Trials of NO donors in man have not been a success and have been limited by systemic side effects, complicated delivery, safety concerns, and failure to show efficacy in human trials (Lablanche et al., 1997; Suzuki et al., 2002; Wohrle et al., 2003; Suzuki et al., 2002; Heiko and Chew, 2006). Recently it has been suggested that nitrite, via its conversion to NO, decreases the development of intimal hyperplasia following vascular injury, an effect also linked with changes in p21<sup>waf1/cip1</sup> expression (Alef et al., 2011). However, whether nitrite might alter cell survival acutely during an ischaemic stimulus, such as primary PCI for AMI or whether nitrite might exert similar repressive effects on endothelial cells during ischaemia, in addition to vascular smooth muscle cells, is uncertain.

The purpose of this study was to investigate the effect of nitrite on the proliferation and viability of endothelial cells (EC) and vascular smooth muscle cells (VSMC) in normoxia, hypoxia and I/R.

## **5.2 Results**

### **5.2.1 Normoxia**

Under normoxic conditions, nitrite treatment for 24h had no significant effect on HCAEC, HUVEC or HmCASM C cell numbers or viability (Figure 5.1).



**Figure 5.1 Nitrite and HCAEC, HUVEC and HmCASM growth under normoxia.**

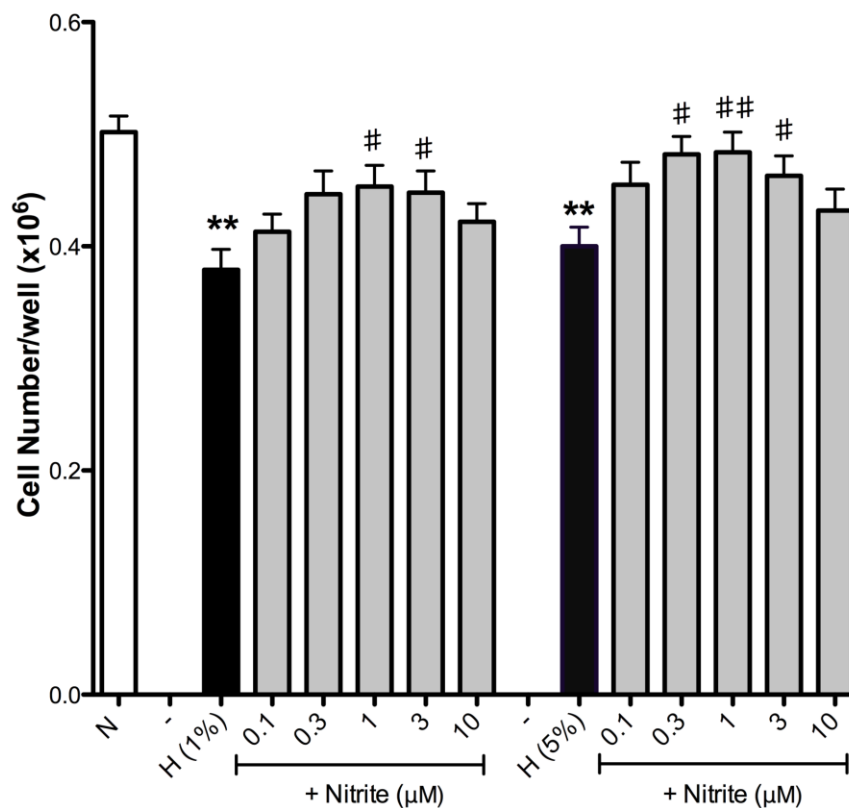
Bar charts showing the effect of nitrite (0.1 -10  $\mu\text{M}$ ) on A) Human coronary artery endothelial cell, B) human umbilical vein cell and C) human coronary artery smooth muscle cell growth under normoxic conditions (21%  $\text{O}_2$ , 5%  $\text{CO}_2$  and 37°C). Data represent means  $\pm$ SEM of (HCAEC n=7, HUVEC, n=13 and HmCASM n=6 independent experiments).  $P > 0.05$  comparing normoxia with 0.1-10 $\mu\text{M}$  of nitrite using one-way ANOVA.

## 5.2.2 Effect of nitrite on cell growth under hypoxic conditions

### 5.2.2.1 HUVECs

#### 5.2.2.1.1 Cell number

In comparison to normoxia, hypoxia of either 5% or 1% attenuated proliferation of HUVECs, with 5% O<sub>2</sub> causing a 21.2±2.8% and 1% O<sub>2</sub> a 24.1±3.1% reduction compared to normoxia (P<0.001, n=10) (Figure 5.2).

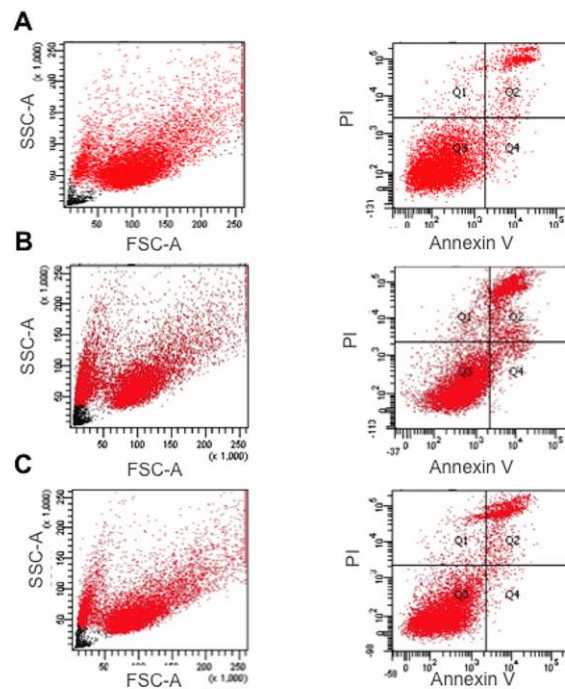


**Figure 5.2 Effect of nitrite on HUVEC growth in hypoxic conditions.**

Effect of nitrite (0.1-10 μM) on human umbilical vein endothelial cell (HUVEC) growth under 5% hypoxia (5% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C) and 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means ±SEM of n=10 independent experiments. N = Normoxia, H = Hypoxia. Significance shown as \*\*P<0.001 comparing Normoxia with hypoxia, #P<0.05, ##P<0.01 comparing the relevant control with nitrite, using 1-way ANOVA, with Bonferroni's multiple comparison test.

### 5.2.2.1.2 Cell Viability

Hypoxia increased the numbers of apoptotic cells, decreasing viability of HUVECs by  $7.4 \pm 1.4\%$  in 5%  $O_2$  and  $9.0 \pm 1.8\%$  at 1%  $O_2$ . ( $P < 0.001$ ,  $n = 10$ ) (Figure 5.3). Nitrite resulted in a concentration-dependent increase in endothelial cell number and viability with a peak effect (near complete reversal) occurring at  $3 \mu M$  in HUVECs ( $P < 0.001$ ,  $n = 10$ ) (Figure 5.2, Table 5.1 and 5.2). This is manifested by a decrease in the % of apoptotic cells with no difference seen in percentage cellular necrosis (Table 5.1/5.2).



**Figure 5.3 HUVEC Viability (5% hypoxia) in the presence of nitrite.**

Representative flow cytometry dot plots of HUVECs in normoxia, or treated with nitrite and exposed to 24 hours 5% hypoxia. Representative FSC versus SSC and the annexin V-FITC versus PI plots are shown. Values in Q1 are positive for PI and negative for annexin and are necrotic cells. Q2 are cells positive for annexin V and PI and are apoptotic. Q3 are negative for both PI and annexin V (Viable), Q4 are early apoptotic (annexin positive and PI negative). Figure A shows plots for cells treated in normoxia for 24 hours. Figure B shows plots for cells treated in 5% hypoxia alone for 24 hours. Figure C shows plots for cells treated with  $3 \mu M$  nitrite in the presence of 5% hypoxia.

Quadrant (%)	Normoxia	Hypoxia (5%)	+ Nitrite				
			0.1µM	0.3µM	1µM	3µM	10µM
1. Necrotic	3.05±1.09	3.27±1.30	3.41±1.19	2.94±1.12	2.52±0.76	1.14±0.20	3.20±1.33
2. Apoptotic	11.54±1.86	17.16±2.78*	16.33±2.32	16.06±2.11	13.72±1.75	13.28±1.77 <sup>#</sup>	15.00±2.01
3. Viable	80.84±1.38	73.41±1.97*	76.50±2.31	76.89±1.84	79.21±1.79	79.25±1.52 <sup>#</sup>	77.19±1.73
4. Early Apoptotic	3.91±0.71	5.09±0.99	3.78±0.95	4.16±0.89	4.53±0.84	4.04±0.94	4.92±1.03

**Table 5.1 HUVEC Viability under 5% hypoxia.**

Effect of nitrite (0.1-10 µM) on HUVEC viability under 5% hypoxia (5% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data expressed as mean±SEM of n=10. Statistical significance shown as \* P<0.05 vs normoxia, # P<0.05 vs 5% hypoxia alone using 1-way ANOVA, with Bonferroni's multiple comparison test.

Quadrant (%)	Normoxia	Hypoxia (1%)	+ Nitrite				
			0.1µM	0.3µM	1µM	3µM	10µM
1. Necrotic	2.74±0.49	2.81±0.66	2.09±0.74	1.64±0.47	2.20±0.46	2.49±0.79	2.55±0.84
2. Apoptotic	8.66±1.03	16.38±2.74*	8.51±1.40	8.39±1.24 <sup>#</sup>	8.91±0.95 <sup>#</sup>	7.70±1.14 <sup>#</sup>	9.73±1.11
3. Viable	85.53±0.98	76.76±2.54*	85.23±1.08	85.76±1.1 <sup>#</sup>	86.19±1.20 <sup>#</sup>	86.78±1.12 <sup>#</sup>	84.19±0.82
4. Early Apop	3.10±0.37	4.06±1.00	4.21±0.73	3.75±0.71	3.15±0.48	3.05±0.53	3.51±0.64

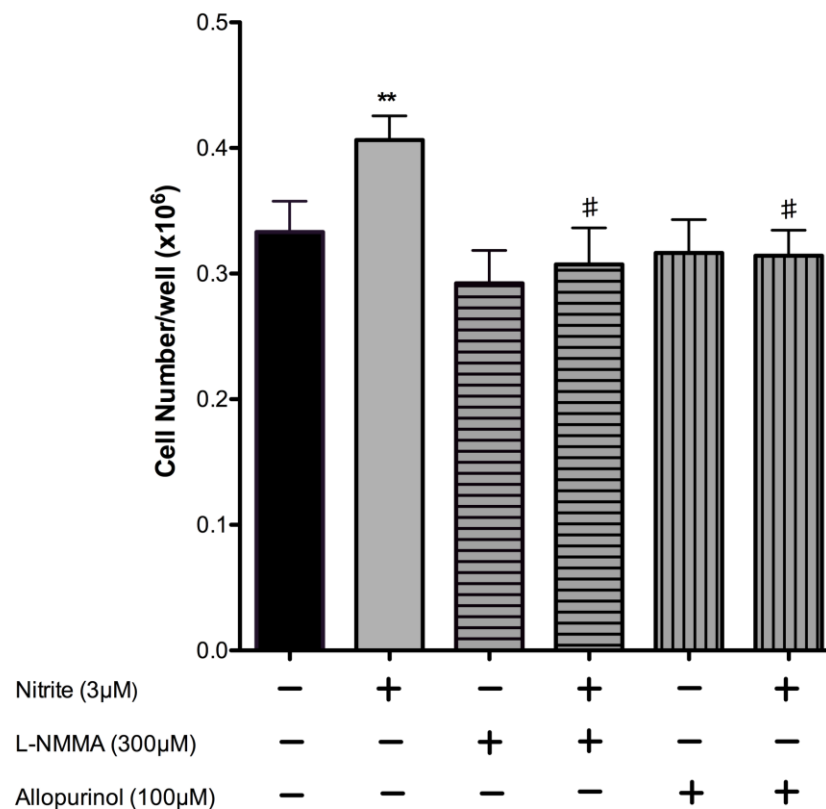
**Table 5.2 HUVEC Viability under 1% hypoxia.**

Effect of nitrite (0.1-10 µM) on HUVEC viability under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data expressed as mean±SEM of n=10. Statistical significance shown as \* P<0.05 vs normoxia, # P<0.05 vs 5% hypoxia alone using 1-way ANOVA, with Bonferroni's multiple comparison test.



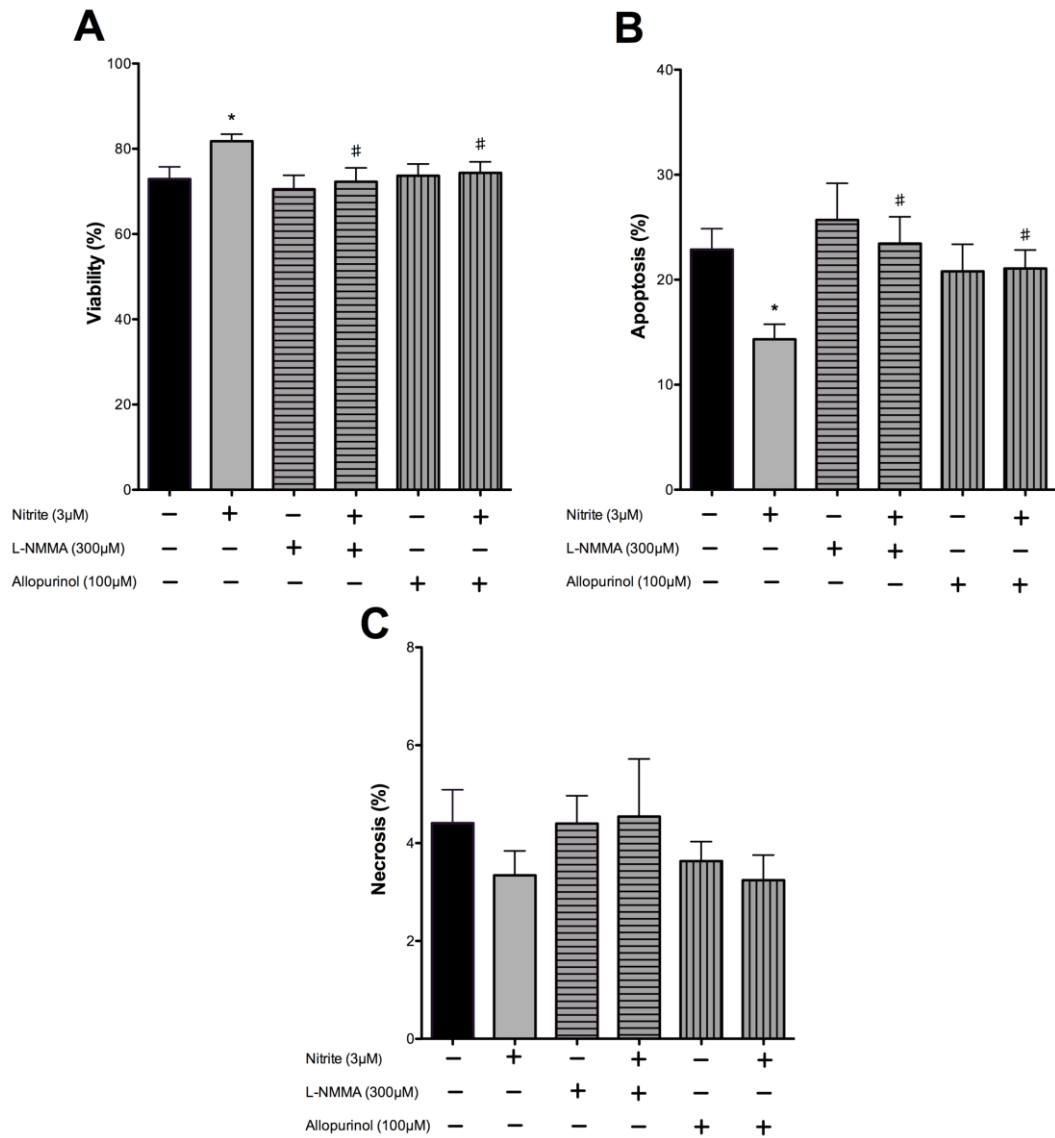
### 5.2.2.1.3 Nitrite reductase inhibitors

To assess the nitrite reductase that might be involved in this effect experiments were conducted using a maximally effective concentration of nitrite under 1% hypoxic conditions. This effect of nitrite (3 $\mu$ M) on cell growth (figure 5.4) and viability (Figure 5.5) was partially attenuated by L-NMMA by ~85% ( $P<0.001$ ,  $n=10$ ) or by treatment with allopurinol (~80.0%,  $P<0.001$ ,  $n=10$ ) (Figure 5.4 and 5.5).



**Figure 5.4 Nitrite and effect of inhibitors (L-NMMA and Allopurinol) on HUVEC growth.**

Nitrite and effect of inhibitors (L-NMMA (300  $\mu$ M) and Allopurinol (100  $\mu$ M)) on human umbilical vein endothelial cell (HUVEC) growth under 1% hypoxia (1%  $O_2$ , 5%  $CO_2$  and 37°C). Data represent means  $\pm$ SEM of  $n=9$  independent experiments. Statistical significance shown as \*\* $P<0.001$  comparing hypoxia with hypoxia + 3 $\mu$ M nitrite, #  $P<0.05$  comparing Hypoxia + 3 $\mu$ M nitrite with hypoxia +3 $\mu$ M nitrite + L-NMMA and allopurinol, using 1-way ANOVA, with Bonferroni's multiple comparison test.



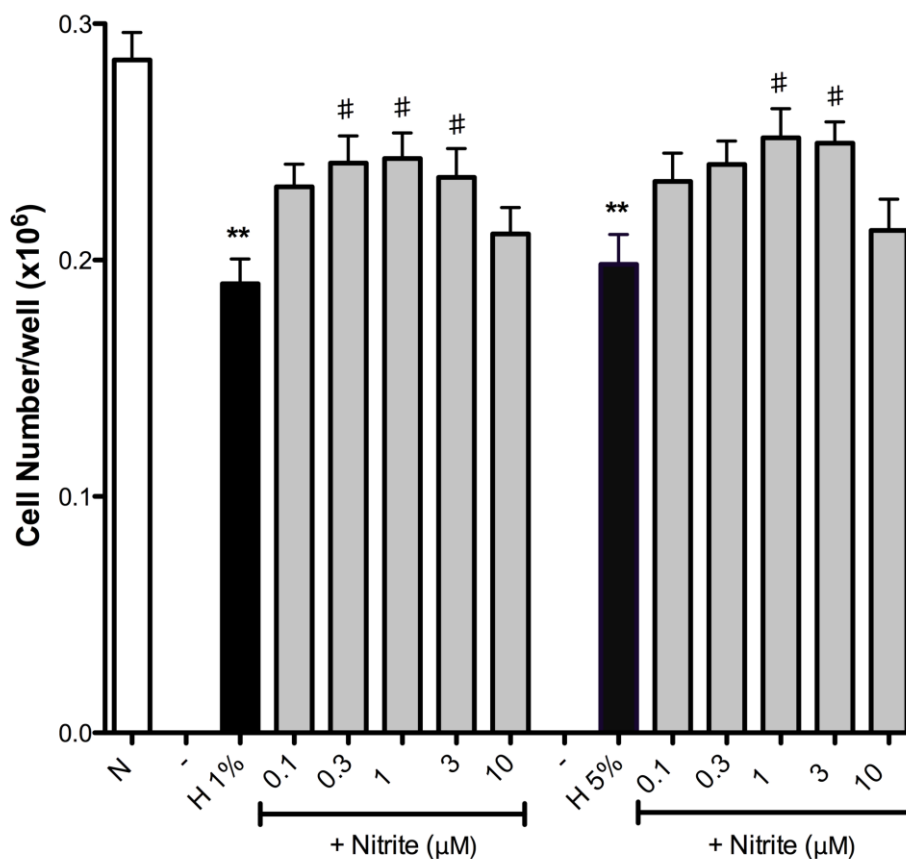
**Figure 5.5 Nitrite and effect of inhibitors (L-NMMA and Allopurinol) on HUVEC viability.**

Nitrite and effect of inhibitors (L-NMMA (300 μM) and Allopurinol (100 μM)) on human umbilical vein endothelial cell (HUVEC) viability under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Figure A shows the percentage (%) viability of HUVEC, B shows the percentage of apoptotic cells and C shows the percentage necrotic cells. Data represent means ±SEM of n=9 independent experiments. Statistical significance shown as \*\*P<0.01 comparing hypoxia with hypoxia + 3μM nitrite, # P<0.05 comparing Hypoxia + 3μM nitrite with hypoxia +3μM nitrite + L-NMMA and allopurinol, using 1-way ANOVA, with Bonferroni's multiple comparison test.

### 5.2.2.2 HCAECs

#### 5.2.2.2.1 Cell number

In comparison to normoxia, hypoxia of either 5% or 1% attenuated proliferation of HCAECs, with 5% O<sub>2</sub> causing a 30.1±4.1% and 1% a 31.4±3.6% reduction (P<0.001, n=10) (Figure 5.6).

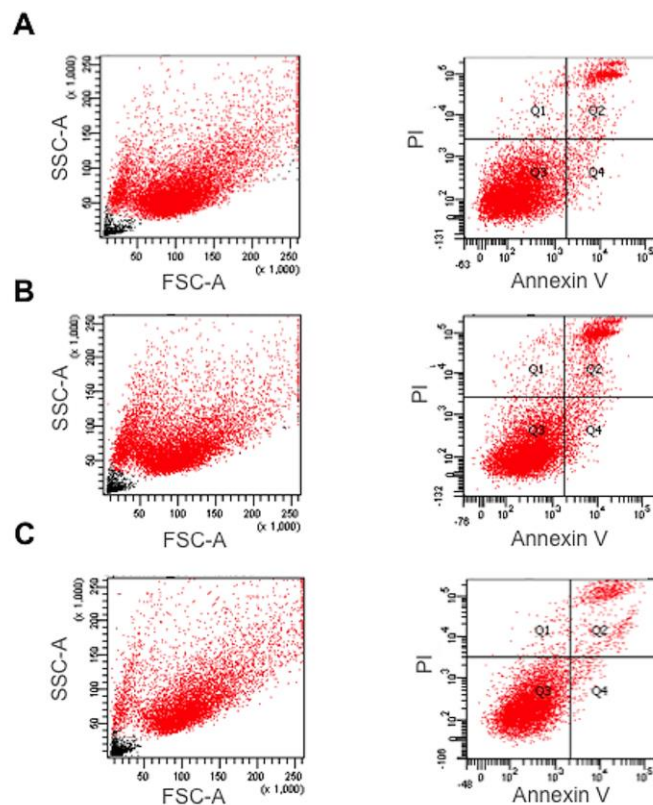


**Figure 5.6 Effect of nitrite on HCAEC growth in hypoxic conditions.**

Effect of nitrite (0.1-10 μM) on human coronary artery endothelial cell (HCAEC) growth under 5% hypoxia (5% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C) and 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means ± SEM of n=10 independent experiments. Statistical significance shown as \*\*P<0.001 comparing normoxia with hypoxia, #P<0.001 comparing the relevant control with nitrite, using 1-way ANOVA, with Bonferroni's multiple comparison test.

#### 5.2.2.2.2 Cell Viability

Hypoxia increased the numbers of apoptotic cells, decreasing viability of HCAECs by  $9.5 \pm 1.9\%$  at 5% and  $9.6 \pm 1.3\%$  at 1% in HCAECs ( $P < 0.001$ ,  $n = 10$ ) (Figure 5.7). Nitrite resulted in a concentration-dependent increase in endothelial cell number and viability with a peak effect (near complete reversal) occurring at  $3 \mu\text{M}$  ( $P < 0.001$ ,  $n = 10$ ) (Table 5.3 and Table 5.4).



**Figure 5.7 HCAEC Viability (1% hypoxia) in the presence of nitrite ( $3 \mu\text{M}$ ).**

Representative flow cytometry dot plots of HCAECs in normoxia and treated with nitrite and exposed to 24 hours 1% hypoxia. Representative FSC versus SSC and the annexin V-FITC versus PI plots are shown. Values in Q1 are positive for PI and negative for annexin and are necrotic cells. Q2 are cells positive for annexin V and PI and are apoptotic. Q3 are negative for both PI and annexin V (Viable), Q4 are early apoptotic (annexin positive and PI negative). Figure A shows plots for cells treated in normoxia for 24 hours. Figure B shows plots for cells treated in 1% hypoxia alone for 24 hours. Figure C shows plots for cells treated with  $3 \mu\text{M}$  nitrite in the presence of 1% hypoxia.

Quadrant (%)	Normoxia	Hypoxia (1%)	+ Nitrite				
			0.1µM	0.3µM	1µM	3µM	10µM
1. Necrotic	2.41±0.57	3.41±1.22	3.93±1.37	2.29 ± 0.42	2.44±0.55	2.66±0.71	2.66±0.56
2. Apoptotic	16.86±1.67	22.03±1.91*	19.23±2.62	19.33±2.44	17.18±2.20 <sup>#</sup>	16.54±1.26 <sup>#</sup>	18.13±2.38
3. Viable	78.04±1.80	68.49±3.00*	73.24±3.55	75.15±2.77 <sup>#</sup>	77.10±2.55 <sup>#</sup>	77.69±2.53 <sup>#</sup>	74.90±3.07
4. Early Apoptotic	2.81±0.38	5.93±2.13	3.51±1.25	3.28±0.99	3.26±0.88	3.09±0.84	4.33±1.26

**Table 5.3 Effect of nitrite on HCAEC viability in 1% hypoxia.**

Effect of nitrite (0.1-10 µM) on HCAEC viability under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C)

Data expressed as mean±SEM of n=10 independent experiments. Statistical significance shown as

\* P<0.05 vs normoxia, # P<0.05 vs hypoxia using 1-way ANOVA, with Bonferroni's multiple comparison test.

Quadrant (%)	Normoxia	Hypoxia (5%)	+ Nitrite				
			0.1µM	0.3µM	1µM	3µM	10µM
1. Necrotic	1.29±0.17	1.67±0.40	1.62±0.29	1.28 ± 0.24	1.30 ± 0.39	1.14 ± 0.20	2.64 ± 0.92
2. Apoptotic	10.43±1.10	14.66±0.89*	12.58±1.01	10.51±1.16	9.52 ± 1.09 <sup>#</sup>	9.88± 1.26 <sup>#</sup>	11.66±0.90
3. Viable	85.20±1.09	76.90±0.94*	80.74±1.02	83.37±1.41	84.47 ± 1.20 <sup>#</sup>	84.67±1.60 <sup>#</sup>	81.08±1.35
4. Early Apoptotic	3.70±0.68	6.74±0.88	5.03±0.82	4.86±0.62	4.70 ± 0.32	4.28 ± 0.73	5.49 ± 0.93

**Table 5.4 Effect of nitrite on HCAEC viability in 5% hypoxia.**

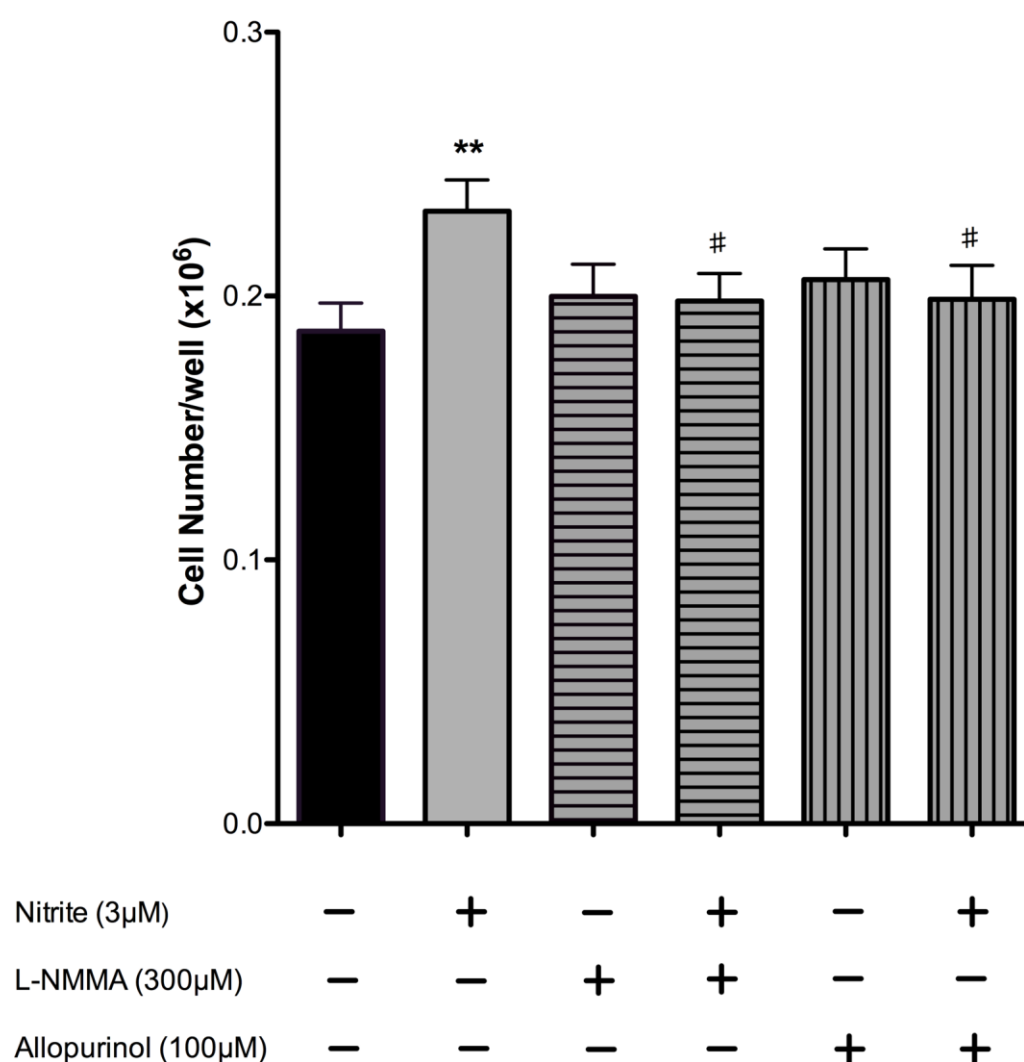
Effect of nitrite (0.1-10 µM) on HCAEC viability under 5% hypoxia (5% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C).

Data expressed as mean±SEM of n=10 independent experiments. Statistical significance shown as

\* P<0.05 vs normoxia, # P<0.05 vs hypoxia using 1-way ANOVA, with Bonferroni's multiple comparison test.

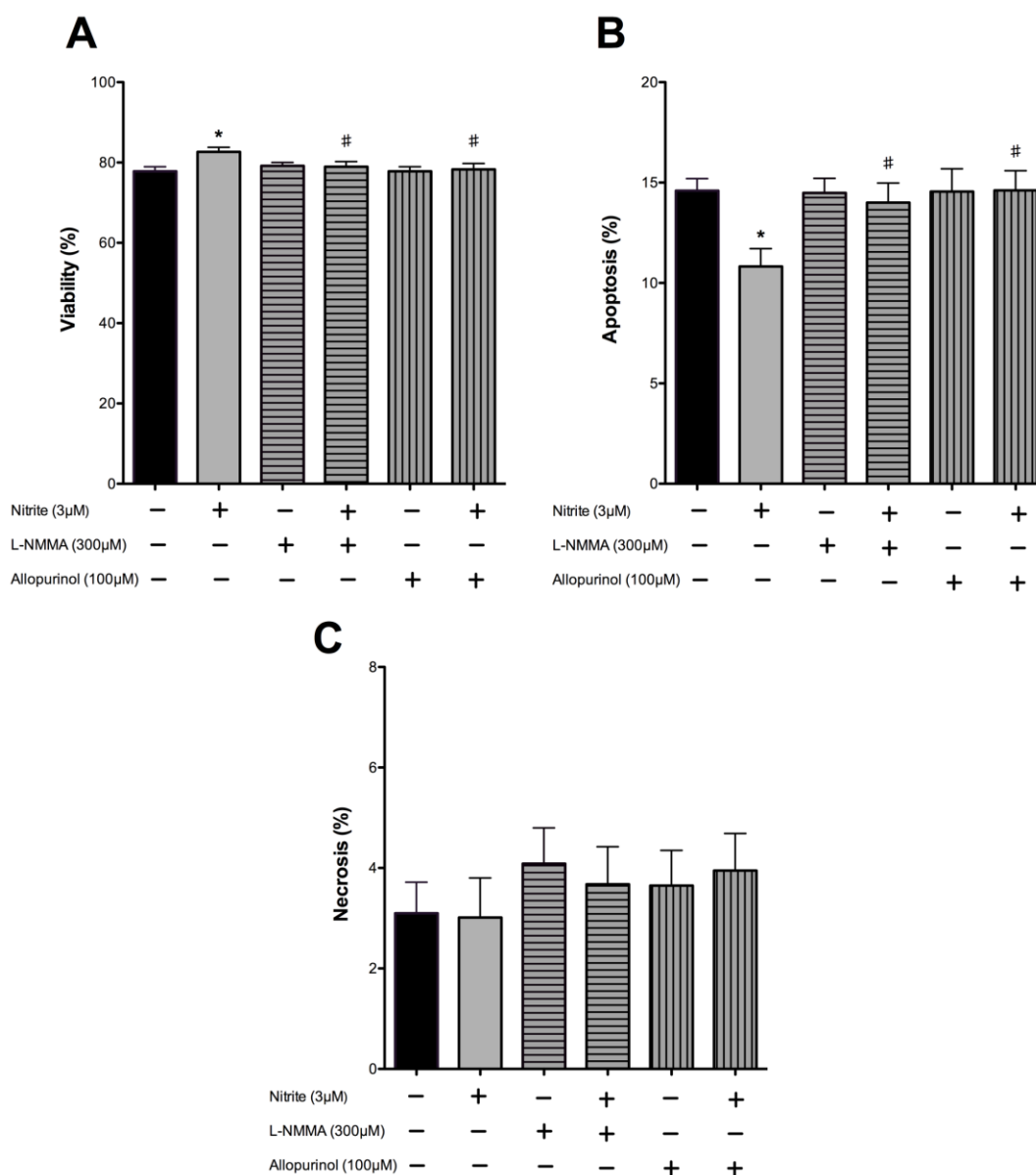
#### **5.2.2.2.3 Nitrite reductase inhibitors**

The effect of nitrite (3 $\mu$ M) on HCAEC growth (figure 5.8) and viability (Figure 5.9) was partially attenuated by L-NMMA by ~75% ( $P<0.001$ ,  $n=10$ ) or by treatment with allopurinol (~70.0%,  $P<0.001$ ,  $n=10$ ) (Figure 5.8). The decrease in apoptosis seen with nitrite (3 $\mu$ M) was reversed by the addition of either inhibitor (Figure 5.9B).



**Figure 5.8. Nitrite and the effect of inhibitors (L-NMMA and Allopurinol) on HCAEC growth.**

Nitrite and the effect of inhibitors (L-NMMA (300μM) and Allopurinol (100μM) on human coronary artery endothelial cell (HCAEC) growth under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means ±SEM of n=9. Statistical significance shown as \*\*P<0.001 comparing hypoxia with hypoxia + nitrite (3μM), #P<0.05 comparing Hypoxia + nitrite (3μM) with hypoxia + nitrite (3μM) + L-NMMA and allopurinol, using 1-way ANOVA, with Bonferroni's multiple comparison test.



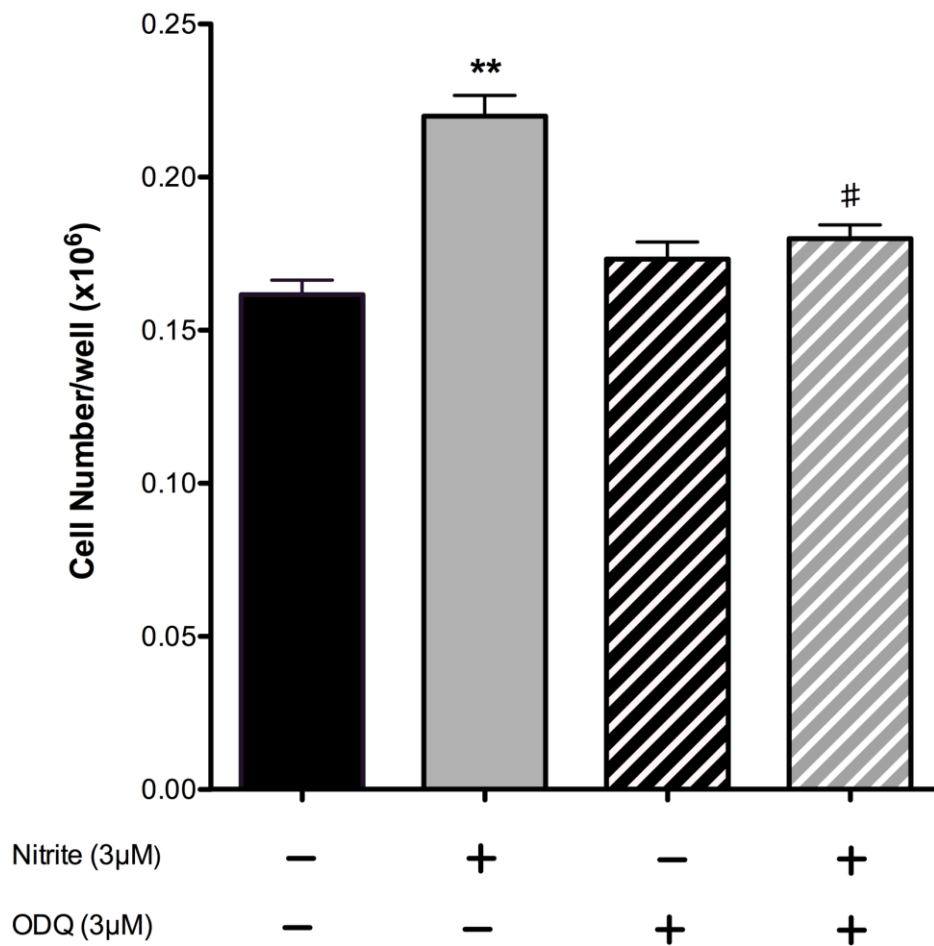
**Figure 5.9 Nitrite and effect of inhibitors (L-NMMA and Allopurinol) on HCAEC viability.**

Nitrite and effect of inhibitors (L-NMMA (300μM) and Allopurinol (100μM) on human coronary artery endothelial cell (HCAEC) viability under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Figure A shows the percentage viability, B shows the percentage of apoptotic cells and C shows the percentage necrotic HCAECs. Data represent means±SEM of n=9 independent experiments. Statistical significance shown as \*\*P<0.01 comparing hypoxia with hypoxia + 3μM nitrite, # P<0.05 comparing Hypoxia + 3μM nitrite with hypoxia +3μM nitrite + L-NMMA and allopurinol, using 1-way ANOVA, with Bonferroni's multiple comparison test.



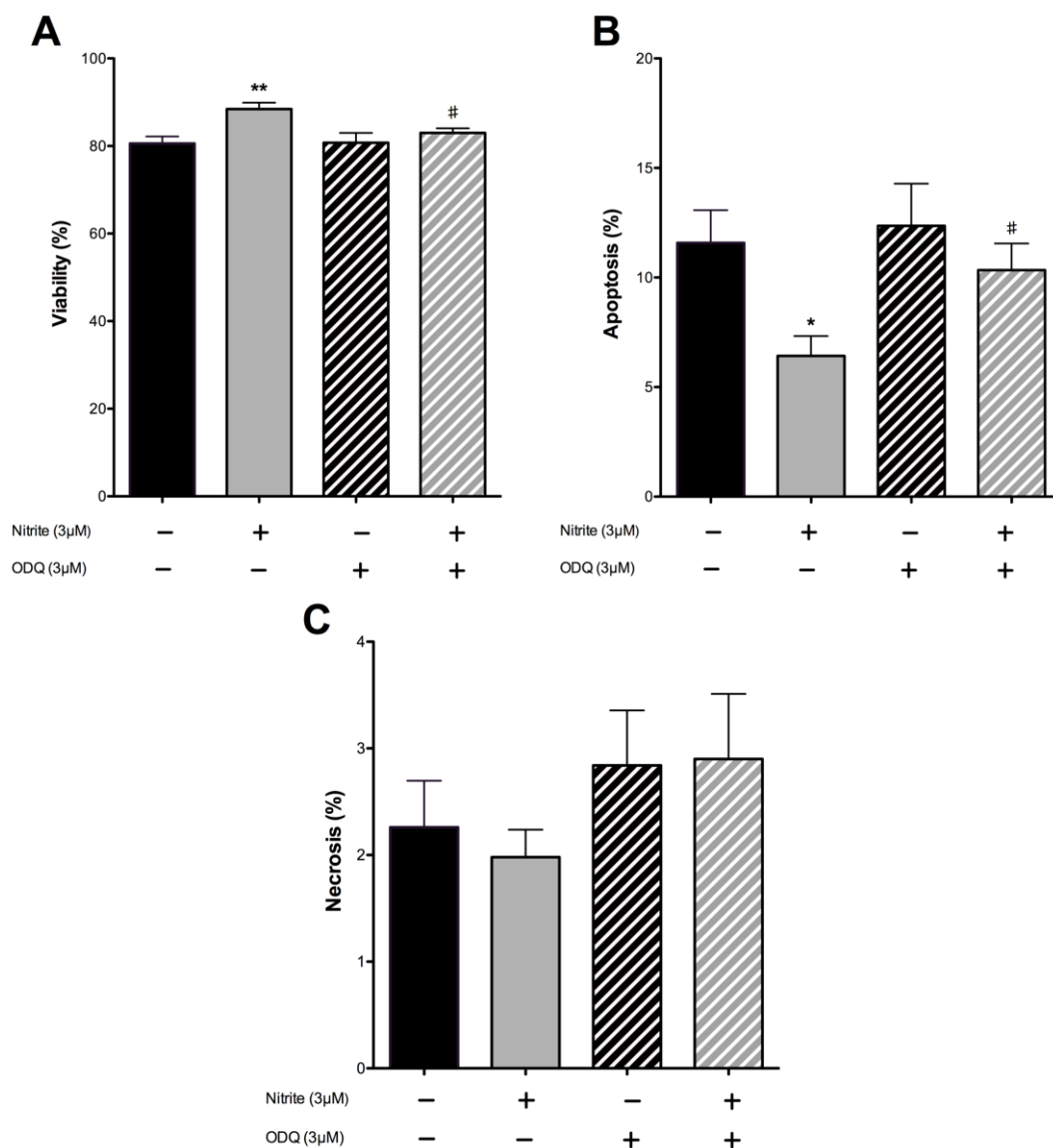
#### 5.2.2.2.4 Effects of sGC inhibition on nitrite-induced effects on endothelial cells

Treatment of cells with the sGC inhibitor ODQ almost abolished the effect of nitrite (3 $\mu$ M) on HCAEC growth (figure 5.10) and viability (Figure 5.11) ( $P < 0.05$ ,  $n = 6$ ). The decrease in apoptosis seen with nitrite (3 $\mu$ M) was reversed by the addition of ODQ (Figure 5.11B).



**Figure 5.10 Nitrite and the effect of sGC inhibitor ODQ.**

Nitrite and the effect of ODQ (3 $\mu$ M) on human coronary artery endothelial cell (HCAEC) growth under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means  $\pm$  SEM of  $n = 6$  independent experiments. Statistical significance shown as \*\* $P < 0.001$  comparing hypoxia with hypoxia + nitrite (3 $\mu$ M), # $P < 0.05$  comparing Hypoxia + nitrite (3 $\mu$ M) with hypoxia + nitrite (3 $\mu$ M) + L-NMMA and allopurinol, using 1-way ANOVA, with Bonferroni's multiple comparison test.

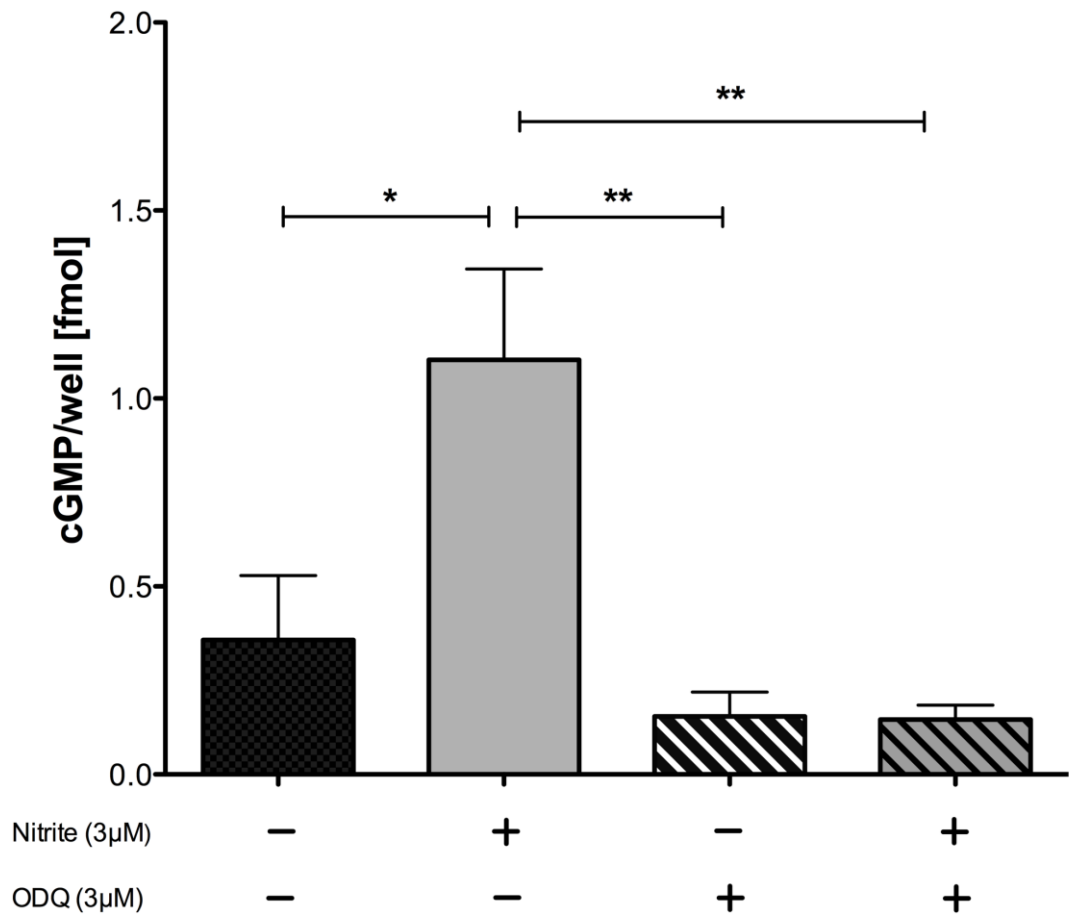


**Figure 5.11. The effect of the sGC inhibitor ODQ on nitrite induced changes in viability of HCAEC**

The effect of the sGC inhibitor ODQ (3μM) on nitrite-induced changes in viability of human coronary artery endothelial cells (HCAEC) under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Figure A shows the percentage viability, B shows the percentage of apoptotic cells and C shows the percentage necrotic HCAECs. Data represent mean±SEM of n=6 independent experiments. Statistical significance shown as \*\*P<0.01 comparing hypoxia with hypoxia + 3μM nitrite, # P<0.05 comparing Hypoxia + 3μM nitrite with hypoxia +3μM nitrite + L-NMMA and allopurinol, using 1-way ANOVA, with Bonferroni's multiple comparison test.

#### 5.2.2.2.5 Cyclic GMP measurement

Levels of cGMP were significantly elevated in HCAECs treated with 3 $\mu$ M nitrite compared to 1% hypoxia control samples ( $P<0.001$ ). This increase in cGMP was reversed by the addition of ODQ (Figure 5.12).

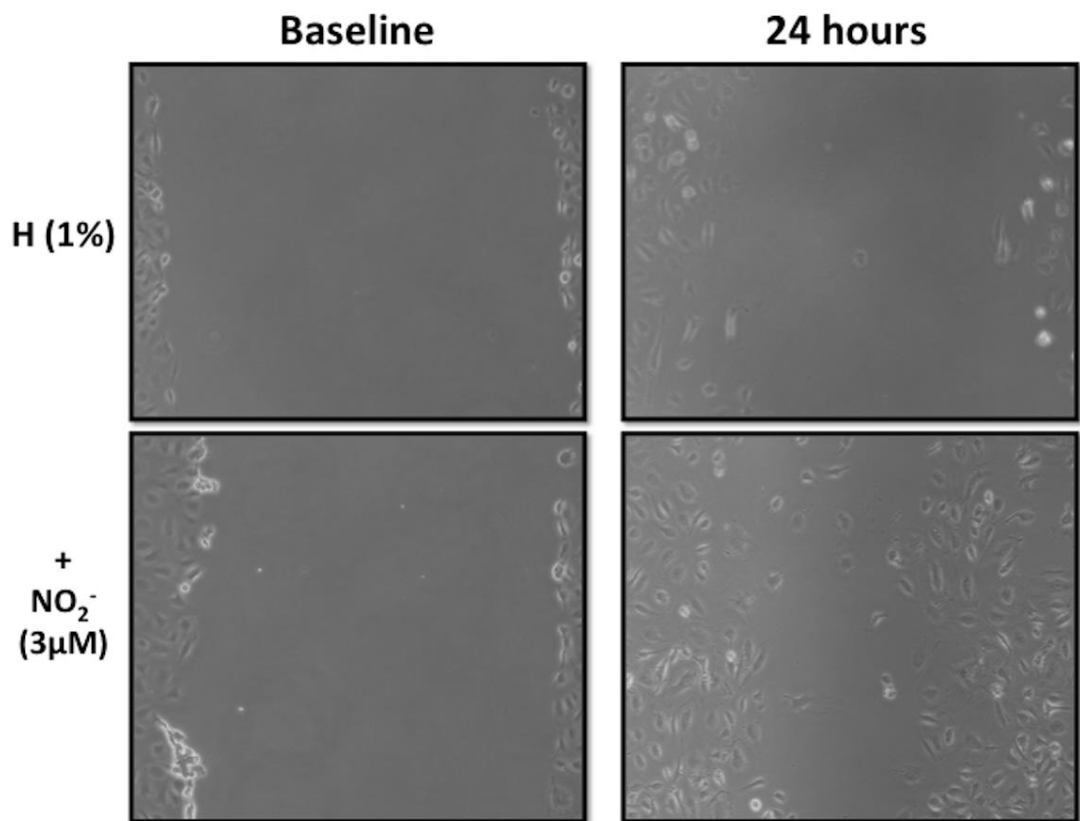


**Figure 5.12 Level of cGMP in HCAECs.**

Effect of 3 $\mu$ M nitrite and ODQ on cGMP levels under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C) Data represent means  $\pm$  SEM of n=4 independent experiments. Statistical significance shown as \*\* $P<0.001$  comparing Normoxia with hypoxia, \* $P<0.05$  comparing the relevant control with nitrite, using 1-way ANOVA, with Bonferroni's multiple comparison test

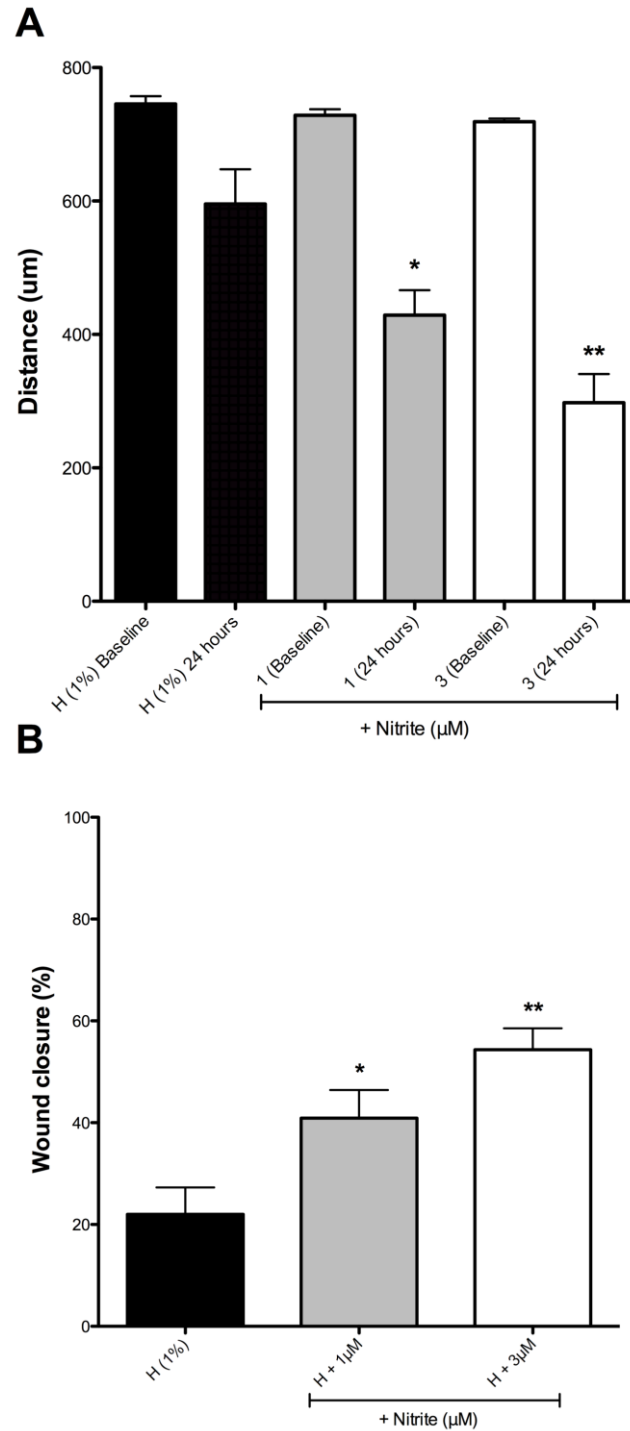
#### 5.2.2.2.6 Nitrite promotes endothelial cell wound closure

To further assess the effects of nitrite on endothelial cell growth scratch wounds were inflicted on cells cultured in 6 well plates with or without nitrite (1 or 3 $\mu$ M) treatment in 1% hypoxia for 24 hours (Figure 5.13). The addition of nitrite resulted in enhanced endothelial cell proliferation and migration compared to control. The wound surface area at 24 hours after treatment was significantly reduced in the nitrite (3 $\mu$ M and 1 $\mu$ M treated) cells in a concentration dependent fashion compared to control (Figure 5.14).



**Figure 5.13 Effect of nitrite on endothelial cell wound healing.**

Example microphotographs of wound closure in control and 3 $\mu$ M nitrite ( $\text{NO}_2^-$ ) treated cells.



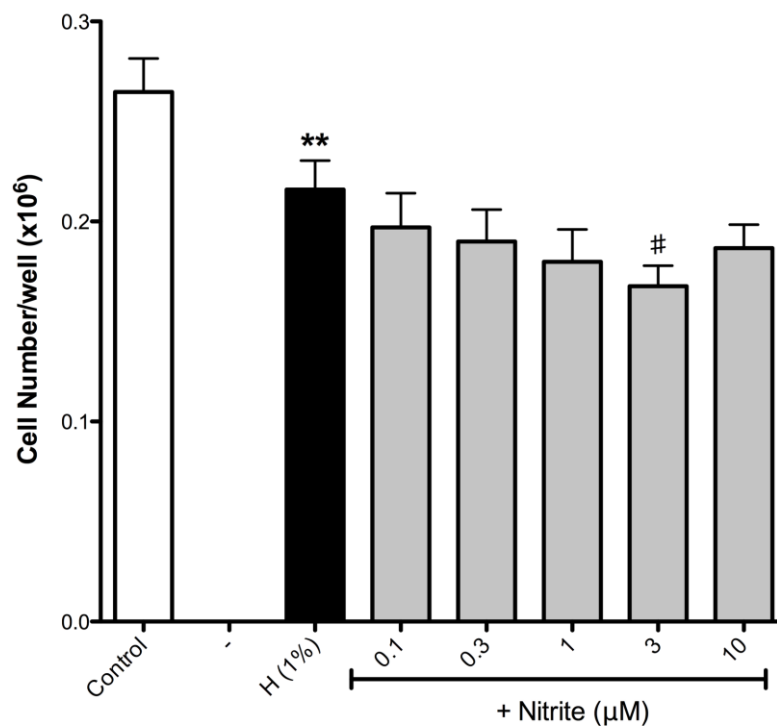
**Figure 5.14 The effect of nitrite on endothelial cell (HCAEC) wound closure.**

Panel A shows the wound size at baseline and 24 hours of treatment (1% hypoxia in presence or absence of nitrite (1 or 3  $\mu\text{M}$ )). Panel B shows the percentage wound closure at 24 hours compared to baseline cells. Data represent mean  $\pm$  SEM of  $n=5$  independent experiments. Statistical significance shown as \*  $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$  comparing hypoxia with hypoxia + nitrite, using 1-way ANOVA, with Bonferroni's multiple comparison test. H=Hypoxia.

### 5.2.2.3 HmCASMCs

#### 5.2.2.3.1 Cell number

Since we saw similar effects of both 1% and 5% O<sub>2</sub> in ECs cell experiments with SMC were conducted in 1% O<sub>2</sub> conditions only. 1% Hypoxia resulted in a significant ( $p < 0.05$ ) decrease in HmCASMC growth compared to normoxia. The addition of nitrite resulted in a concentration-dependent decrease in cell growth compared to control under hypoxic conditions with the greatest effect occurring at 3  $\mu$ M nitrite (Fig 5.15).



**Figure 5.15 Nitrite and HmCASMC growth (1% hypoxia).**

Effect of nitrite (0.1-10  $\mu$ M) on human coronary artery smooth muscle cell (HmCASMC) growth under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means  $\pm$  SEM of  $n=7$  independent experiments. Statistical significance shown as \*\*  $p < 0.05$  comparing Normoxia with 1% hypoxia, # $p < 0.05$  comparing 1% hypoxia with 3  $\mu$ M nitrite, using 1-way ANOVA, with Bonferroni's multiple comparison test. H=Hypoxia

### 5.2.2.3.2 Cell Viability

1% hypoxia had no effect on HCASMC viability or the apoptotic cell number (Table 5.5) and nitrite treatment did not alter this.

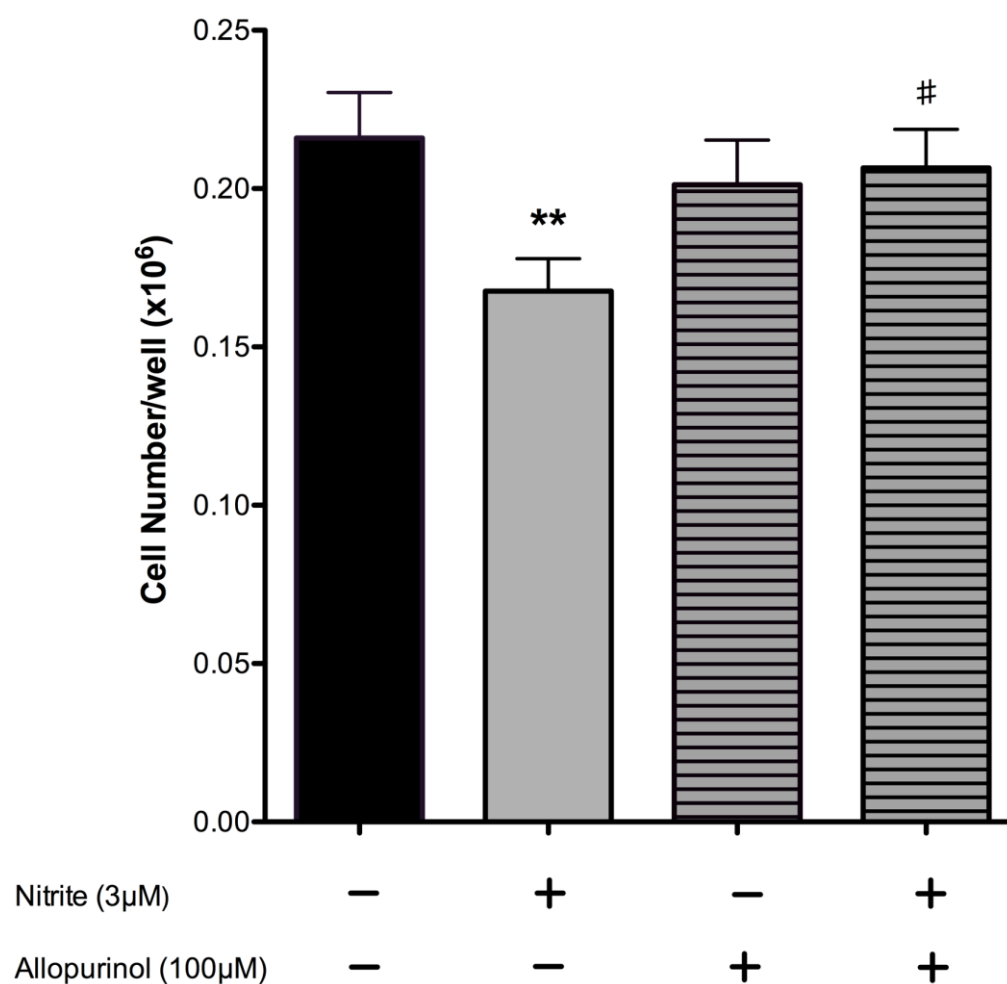
Mechanism (Quadrant)	Normoxia	Hypoxia	+Nitrite				
			0.1µM	0.3µM	1µM	3µM	10µM
Necrosis	0.74 ± 1.03	1.58 ± 0.30	1.24 ± 0.39	1.36 ± 0.49	1.29±0.25	1.74±0.53	1.36±0.49
Apoptosis	1.20 ±0.97	1.40 ± 0.18	1.53 ± 0.38	1.70±0.46	1.61±0.32	2.04±0.42	2.10±0.46
Viable	97.61 ±1.98	95.61 ± 1.98	96.69 ± 0.89	96.46±1.06	96.41±0.81	95.61±1.06	95.16±1.06
Early apoptosis	0.65 ±0.13	0.54 ± 0.16	0.48 ± 0.16	0.48 ± 0.16	0.73±0.16	0.61±0.17	1.38±1.25

**Table 5.5 Cell viability of HmCASMCs under 1% hypoxia.**

Data expressed as mean±SEM of n=7 independent experiments. Statistical significance shown as \* P<0.05 vs normoxia, # P<0.05 vs hypoxia using 1-way ANOVA, with Bonferroni's multiple comparison test.

### 5.2.2.3.3 Nitrite reductase inhibitors

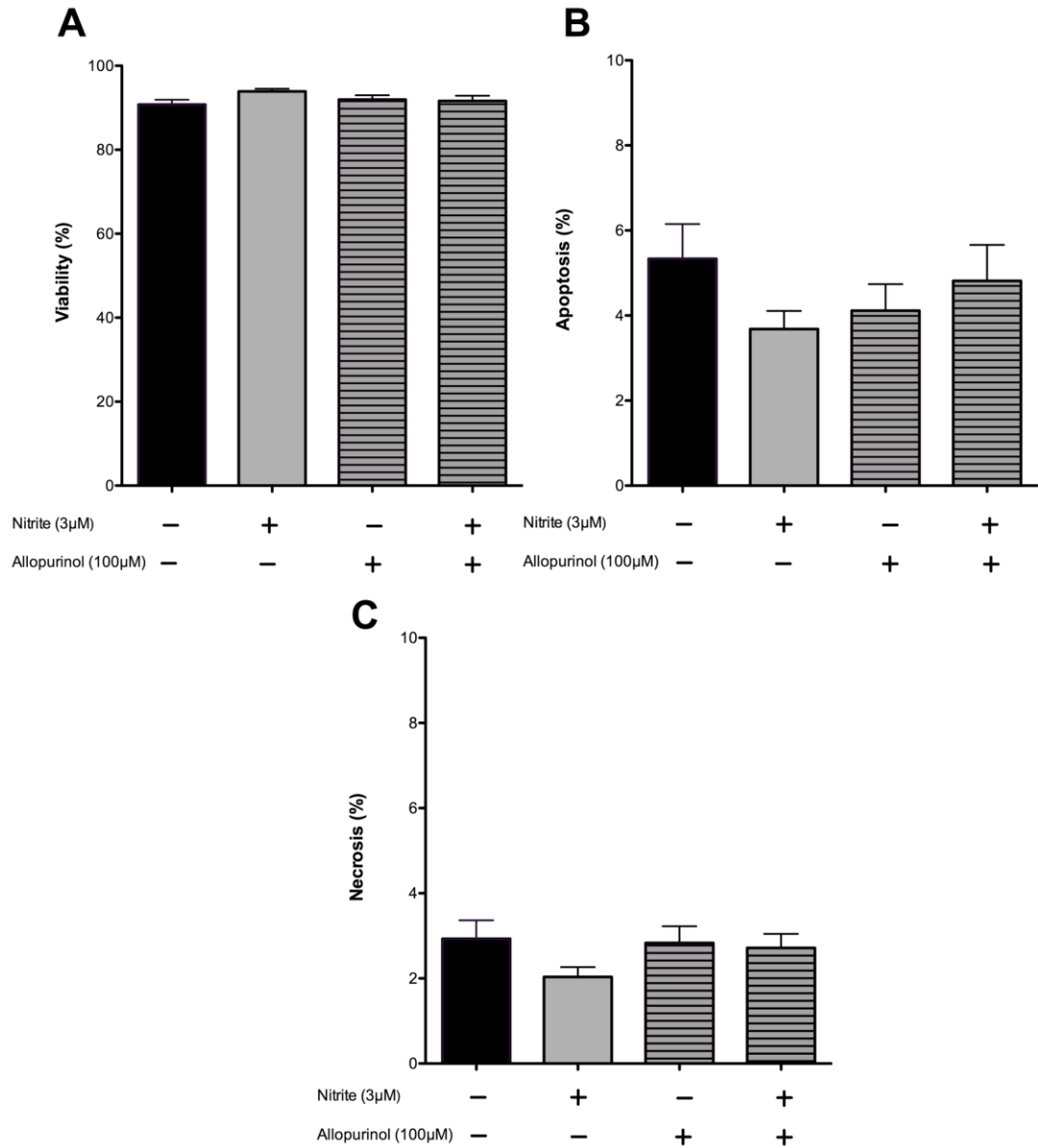
The effect of nitrite (3µM) on HmCASMC growth (Figure 5.16) was significantly attenuated by treatment with allopurinol (~70.0%, P<0.001, n=10). No difference was seen in cell viability (Figure 5.17). The effects of L-NMMA were not assessed since eNOS is not expressed in these cells.



**Figure 5.16 Nitrite and the effect of Allopurinol on HmCASM cell growth.**

Nitrite and the effect of allopurinol on human coronary artery smooth muscle cell (HmCASM) growth under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means ±SEM of n=6 independent experiments. Statistical significance shown as \*\*P<0.05 comparing hypoxia with hypoxia + nitrite (3μM), #P<0.05 comparing Hypoxia + nitrite (3μM) with hypoxia + nitrite (3μM) + allopurinol, using 1-way ANOVA, with Bonferroni's multiple comparison test.



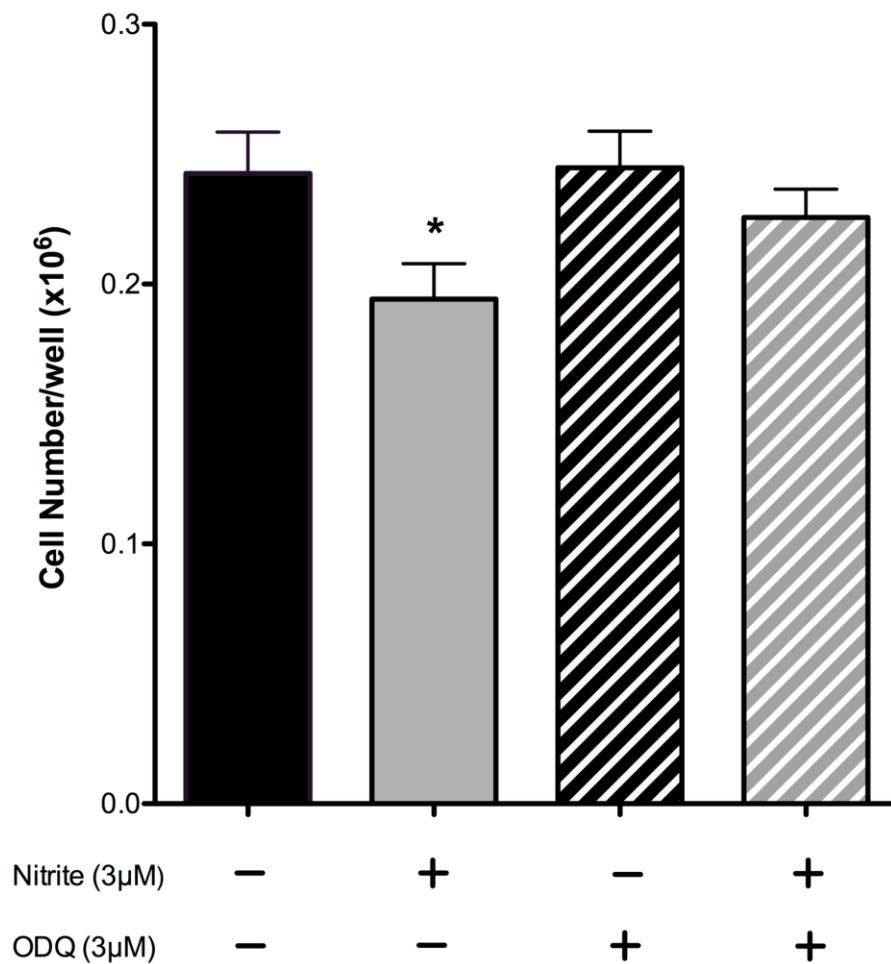


**Figure 5.17 Nitrite and the effect of Allopurinol on HmCASM cell viability.**

Nitrite and the effect of Allopurinol on human coronary artery smooth muscle cell (HmCASM) growth under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Figure A shows the percentage cell viability, B shows the percentage of apoptotic cells and C shows the percentage necrotic HmCASMCs. Data represent mean±SEM of n=6 independent experiments. No differences were seen between treatment groups. Statistical comparison made using 1-way ANOVA, with Bonferroni's multiple comparison test.

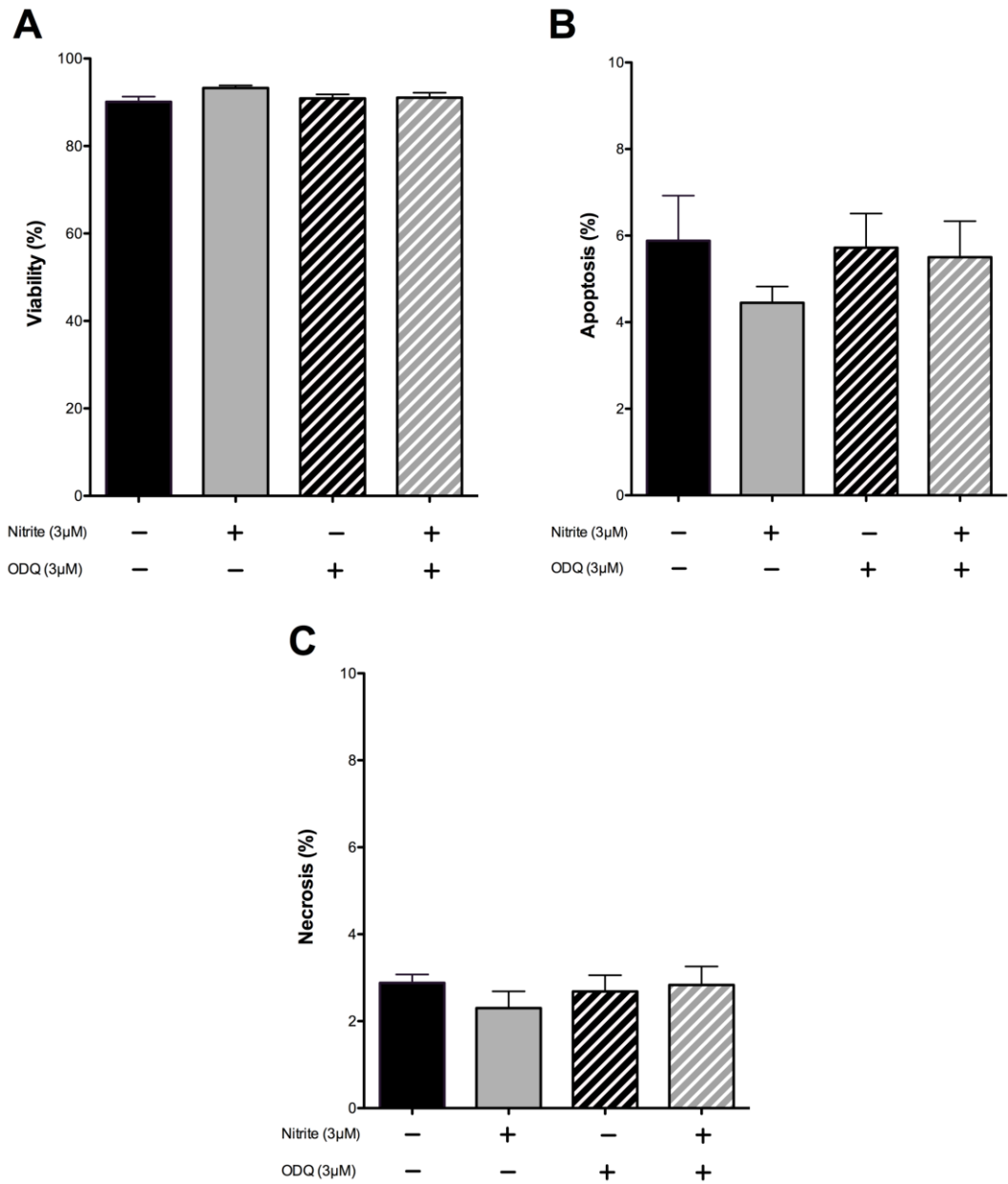
### 5.2.2.3.3 Effects of sGC inhibition on nitrite-induced effects on smooth muscle cells

The effect of nitrite (3 $\mu$ M) on HmCASMC growth (Figure 5.18) was partially attenuated by the sGC inhibitor ODQ by ~58% ( $P<0.05$ ,  $n=6$ ). No difference in the viability of HmCASC was seen with the addition of ODQ (Figure 5.19).



**Figure 5.18 Nitrite and the effect of sGC inhibitor ODQ on HmCASMC growth.**

Nitrite and the effect of ODQ (3 $\mu$ M) on human coronary smooth muscle cell (HmCASMC) growth under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means  $\pm$ SEM of  $n=6$  independent experiments. Statistical significance shown as \* $P<0.05$  comparing hypoxia with hypoxia + nitrite (3 $\mu$ M), using 1-way ANOVA, with Bonferroni's multiple comparison test.

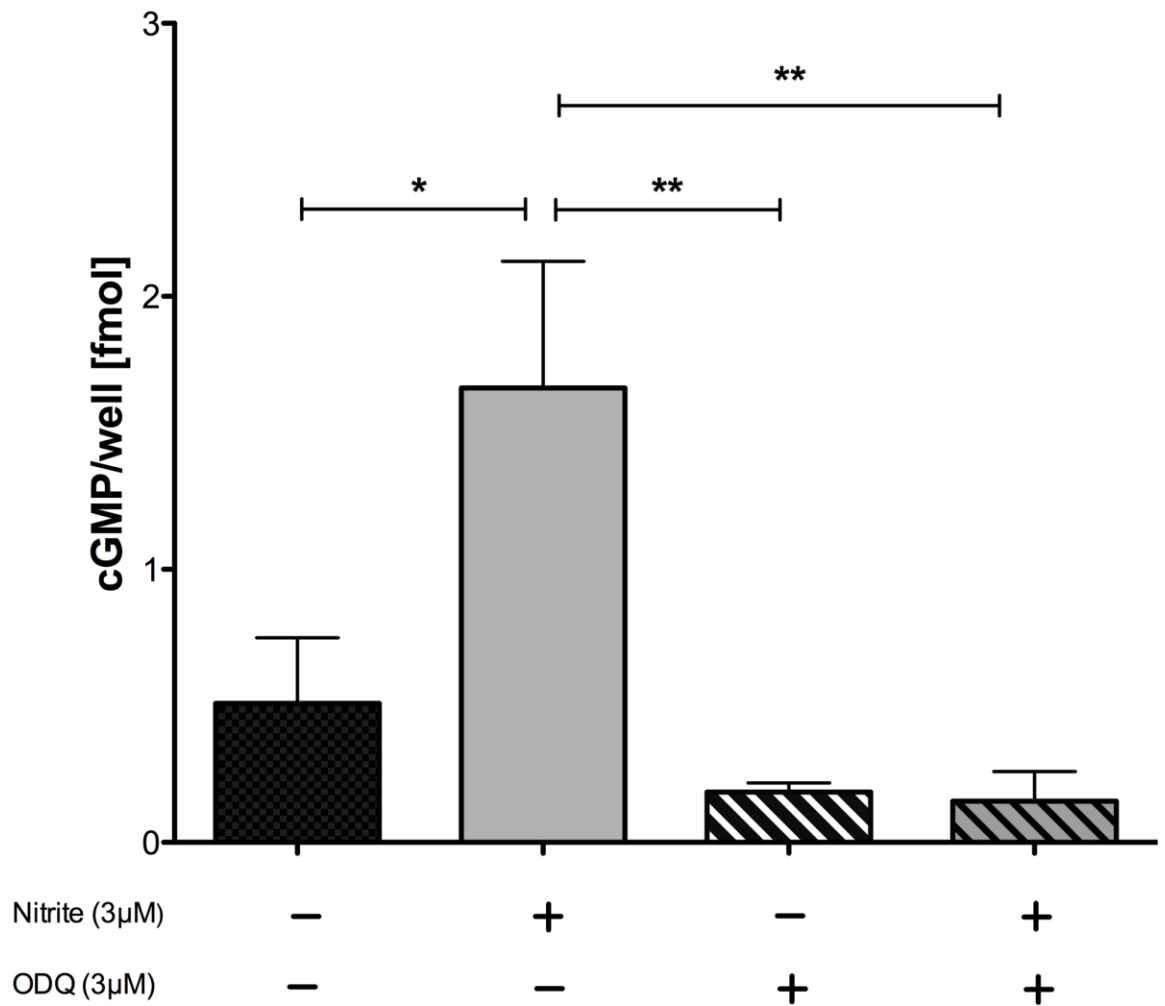


**Figure 5.19 The effect of the sGC inhibitor ODQ on nitrite induced changes in viability of HmCASMC.**

The effect of the sGC inhibitor ODQ (3μM) on nitrite induced changes in viability of human coronary artery smooth muscle cells (HmCASMC) under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Figure A shows the percentage viability, B shows the percentage of apoptotic cells and C shows the percentage necrotic HmCASMCs. Data represent mean±SEM of n=6 independent experiments. No differences seen between treatment groups. Statistical comparison made using 1-way ANOVA, with Bonferroni's multiple comparison test.

#### 5.2.2.3.4 Cyclic GMP measurement

Levels of cGMP were significantly elevated in HmCAsMCs treated with 3 $\mu$ M nitrite compared to 1% hypoxia control samples ( $P<0.001$ ). This increase in cGMP was reversed by the addition of ODQ (Figure 5.20).

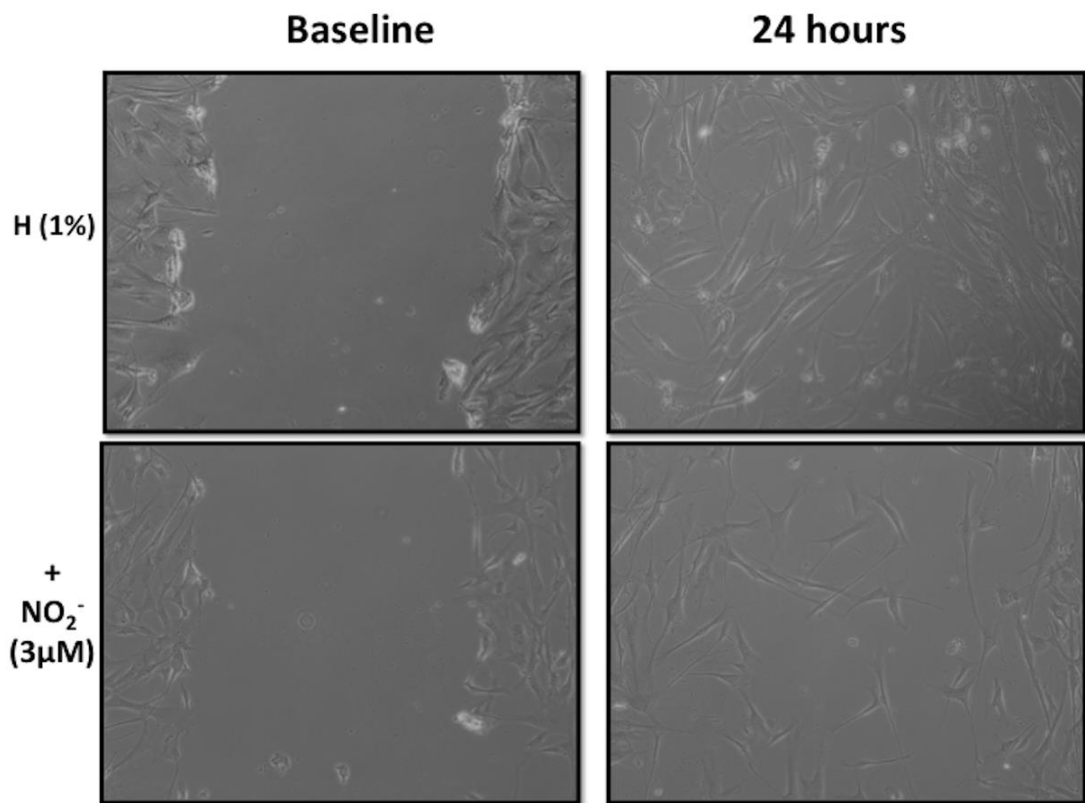


**Figure 5.20 Cyclic GMP levels in HmCAsMCs.**

Effect of 3 $\mu$ M nitrite and ODQ (3 $\mu$ M) on cGMP levels under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means  $\pm$  SEM of  $n=5$  independent experiments. Statistical significance shown as \*\* $P<0.001$  comparing Normoxia with hypoxia, \* $P<0.05$  comparing the relevant control with nitrite, using 1-way ANOVA, with Bonferroni's multiple comparison test.

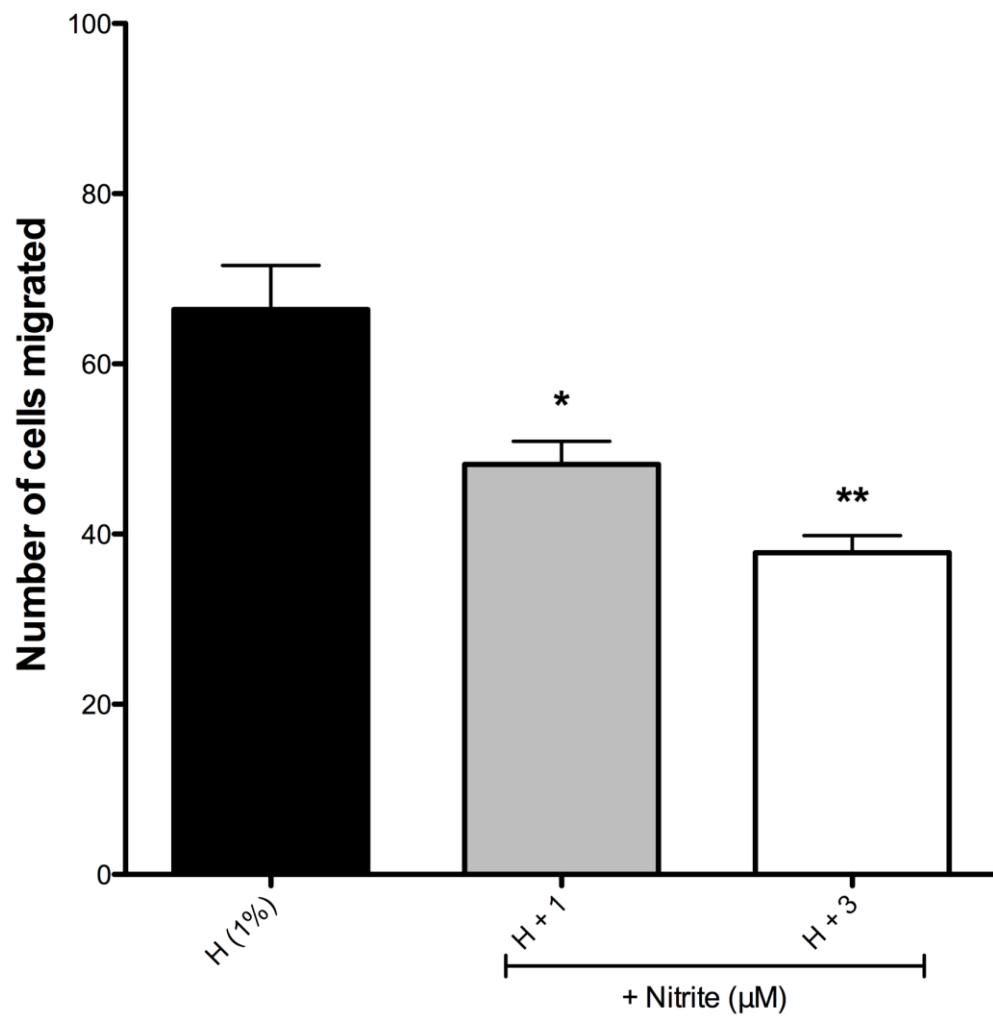
#### 5.2.2.3.5 Nitrite inhibits smooth muscle cell proliferation

Scratch wounds were inflicted on HmCASCs treated with or without nitrite (1 or 3 $\mu$ m) in 1% hypoxia for 24 hours (Figure 5.21). The addition of nitrite resulted in decreased smooth muscle cell proliferation and migration compared to control. The number of cells migrated into the wound area at 24 hours after treatment was significantly reduced in the nitrite treated cells in a concentration dependent fashion compared to control ( $p=0.0003$ ) (Figure 5.22).



**Figure 5.21 Effect of nitrite on smooth muscle cell wound healing.**

Example microphotographs of wound closure in control and 3 $\mu$ m nitrite treated cells.



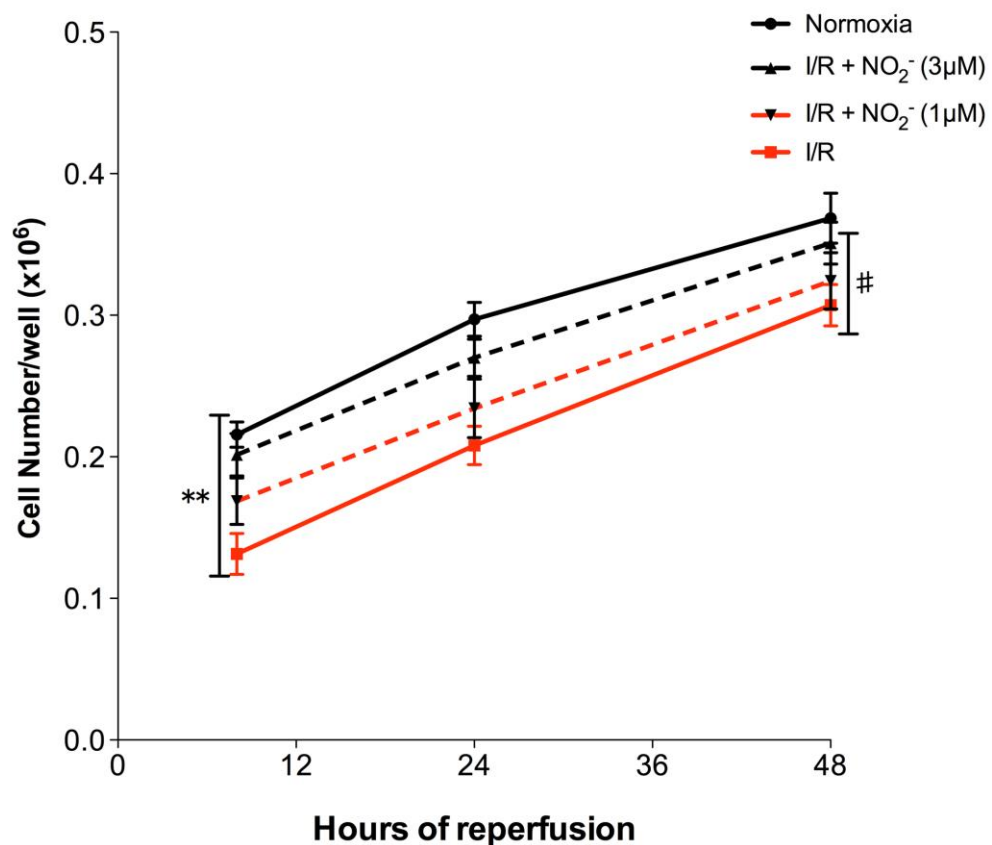
**Figure 5.22 Nitrite and the effect on smooth muscle cell (HmCASM) wound healing.**

The effect of nitrite (1 or 3  $\mu\text{M}$ ) on HmCASM migration and proliferation tested with scratch assay. Data represent mean  $\pm$  SEM of  $n=5$  independent experiments. Statistical significance shown as \*  $P<0.05$ , \*\* $P<0.01$  comparing hypoxia with hypoxia + nitrite, using 1-way ANOVA, with Bonferroni's multiple comparison test. H=Hypoxia.

### 5.3 I/R Injury

#### 5.3.1 HCAEC

Compared to normoxia, EC growth following I/R was significantly attenuated. Restoration of O<sub>2</sub> did not recover cell numbers to normoxia levels at any time point. The addition of nitrite (1 or 3  $\mu$ M) resulted in a significant concentration dependent improvement in cell growth compared to hypoxia alone (Figure 5.23).



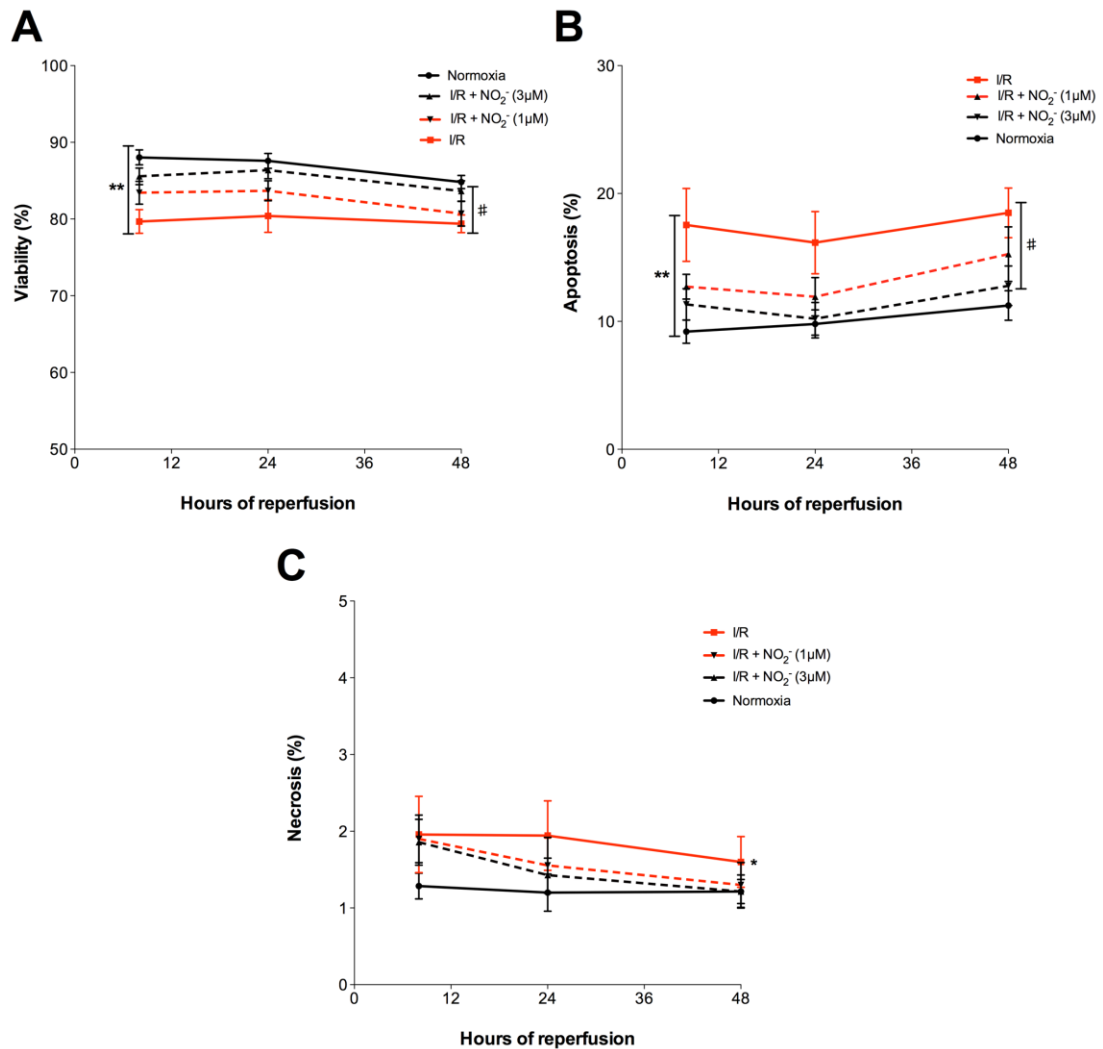
**Figure 5.23 Effect of nitrite on HCAEC cell growth following I/R injury.**

Effect of nitrite on HCAEC cell growth following I/R injury (24h 0.1% hypoxia and the presence or absence of nitrite (1 or 3  $\mu$ M) during re-oxygenation (21% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C) at 8, 24 and 48 hours. Data represent means  $\pm$  SEM of n=7 independent experiments. Statistical significance shown as \*\*P<0.001 comparing normoxia with I/R, and # for p<0.001 and comparing I/R with I/R + nitrite using 2-way ANOVA.

#### **5.3.1.1 Cell Viability**

Following ischaemia, cell viability was significantly attenuated in comparison to normoxia caused by an increase in apoptotic cell number (Figure 5.24) as shown previously. Restoration of O<sub>2</sub> did not recover cell viability to normoxic levels at any time point up to 48-hours post reperfusion. The addition of nitrite (1 or 3 µM) resulted in a significant concentration-dependent improvement in cell viability as a result of decreased apoptosis compared to hypoxia alone (Figure 5.24). No differences were seen between necrotic cell percentages between nitrite treated and hypoxia alone, although necrotic cell numbers were higher in hypoxia compared to normoxia (Figure 5.24).



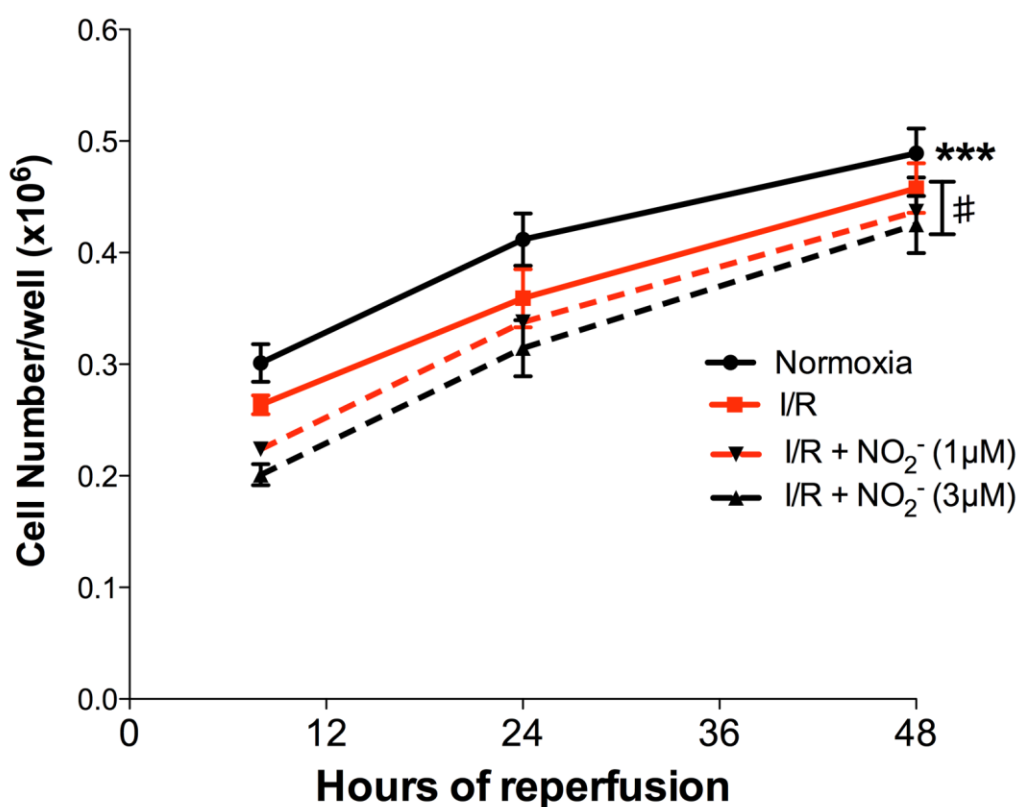


**Figure 5.24 Effect of nitrite on HCAEC viability following I/R injury.**

Effect of nitrite on HCAEC cell viability following I/R injury (24h 0.1% hypoxia) and the presence or absence of nitrite (1 or 3  $\mu\text{M}$ ) during re-oxygenation (21%  $\text{O}_2$ , 5%  $\text{CO}_2$  and  $37^\circ\text{C}$ ) at 8, 24 and 48 hours. Figure A shows the percentage cell viability, B shows the percentage of apoptotic cells and C shows the percentage necrotic HCAECs. Data represent mean $\pm$ SEM of  $n=7$  independent experiments. Statistical significance shown as \* $P<0.05$ , \*\* $P<0.001$  comparing normoxia with I/R, and # for  $p<0.001$  and comparing I/R with I/R + nitrite using 2-way ANOVA. I/R=Ischaemia/Reperfusion.

### 5.3.2 HmCASC

Compared to normoxia, HmCASC growth following I/R was significantly ( $P<0.001$ ) inhibited. The addition of  $1\mu\text{M}$  and  $3\mu\text{M}$  nitrite significantly decreased cell numbers compared to I/R alone following reperfusion (Figure 5.25).

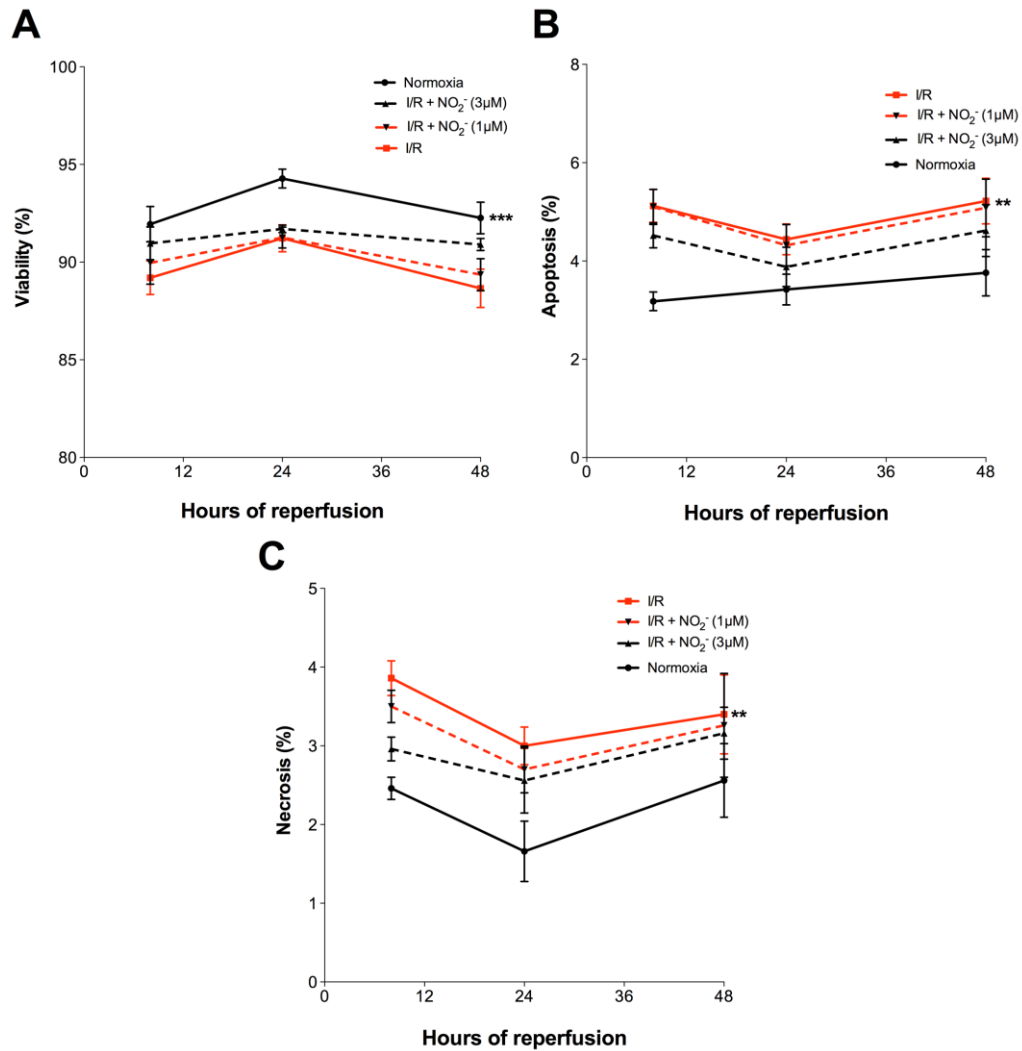


**Figure 5.25 Effect of nitrite on HmCASC growth following I/R injury.**

Effect of nitrite on HmCASC growth following I/R injury (24h 0.1% hypoxia) and the presence or absence of nitrite (1 or 3  $\mu\text{M}$ ) during re-oxygenation (21% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C) at 8, 24 and 48 hours. Data represent means  $\pm$  SEM of  $n=5$  independent experiments. Statistical significance shown as \*\*\* $P<0.001$  comparing normoxia with I/R, and # for  $p<0.001$  and comparing I/R with I/R + nitrite using 2-way ANOVA.

### **5.3.2.1 Cell Viability**

Following ischaemia, cell viability was significantly attenuated in comparison to normoxia caused by an increase in apoptotic cell number (Figure 5.26). Restoration of O<sub>2</sub> did not recover cell viability to normoxia levels at any time point. Despite the addition of nitrite (1 or 3 µM) being associated with a significant decrease in cell number, there was no significant effect on cell viability with no difference in rates of apoptosis or necrosis between nitrite treated and I/R control (Figure 5.26).



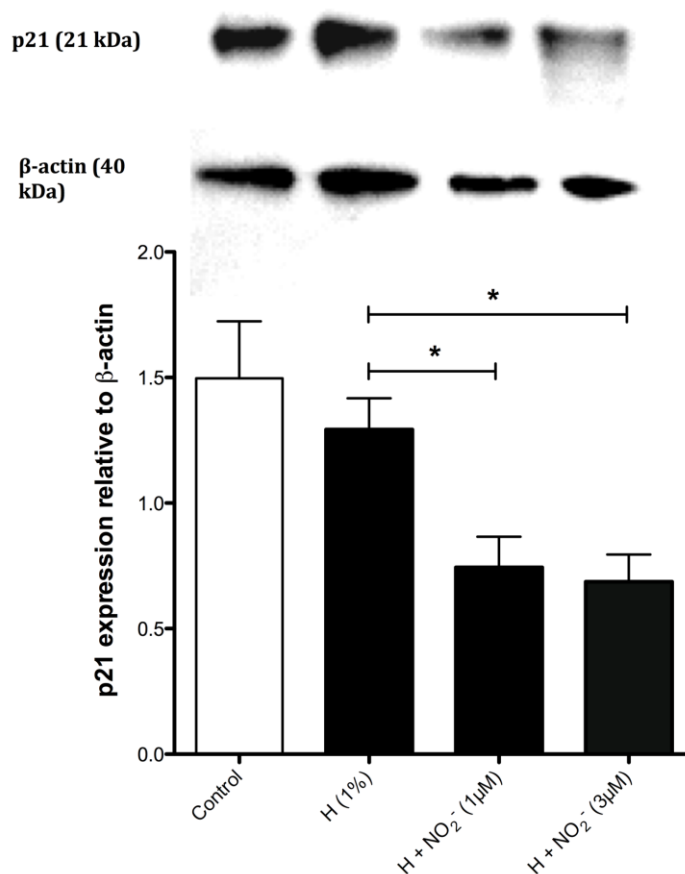
**Figure 5.26 Effect of nitrite on HmCASC viability following I/R injury.**

Effect of nitrite on HmCASC viability following I/R injury (24h 0.1% hypoxia) and the presence or absence of nitrite (1 or 3  $\mu$ M) during re-oxygenation (21% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C) at 8, 24 and 48 hours. Figure A shows the percentage cell viability, B shows the percentage of apoptotic cells and C shows the percentage necrotic HmCASCs. Data represent mean $\pm$ SEM of n=5 independent experiments. Statistical significance shown as \*P<0.05, \*\*P<0.001 comparing normoxia with I/R, using 2-way ANOVA. I/R=Ischaemia/Reperfusion.

## 5.4 Cell cycle markers

### 5.4.1 HCAEC

Nitrite decreased p21 protein levels in a concentration-dependent fashion under 1% hypoxia with a significant decrease in p21 levels with 3 $\mu$ M and 1 $\mu$ M nitrite treatment compared to control conditions ( $P<0.05$ ) (Figure 5.27)

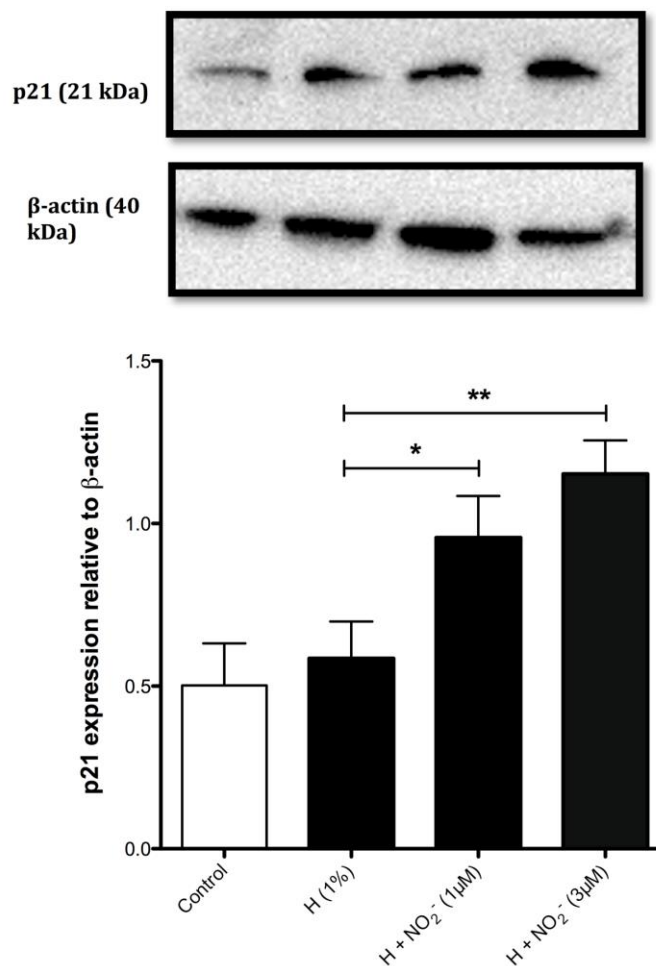


**Figure 5.27 Effect of nitrite (1 and 3  $\mu$ M) on p21 expression in HCAECs.**

Nitrite and the effect on p21 expression in human coronary artery endothelial cells (HCAEC) under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means  $\pm$ SEM of n=5 independent experiments. Statistical significance shown as \* $P<0.05$  comparing hypoxia with hypoxia + nitrite (3 $\mu$ M or 1 $\mu$ M), using 1-way ANOVA, with Bonferroni's multiple comparison test. H=Hypoxia.

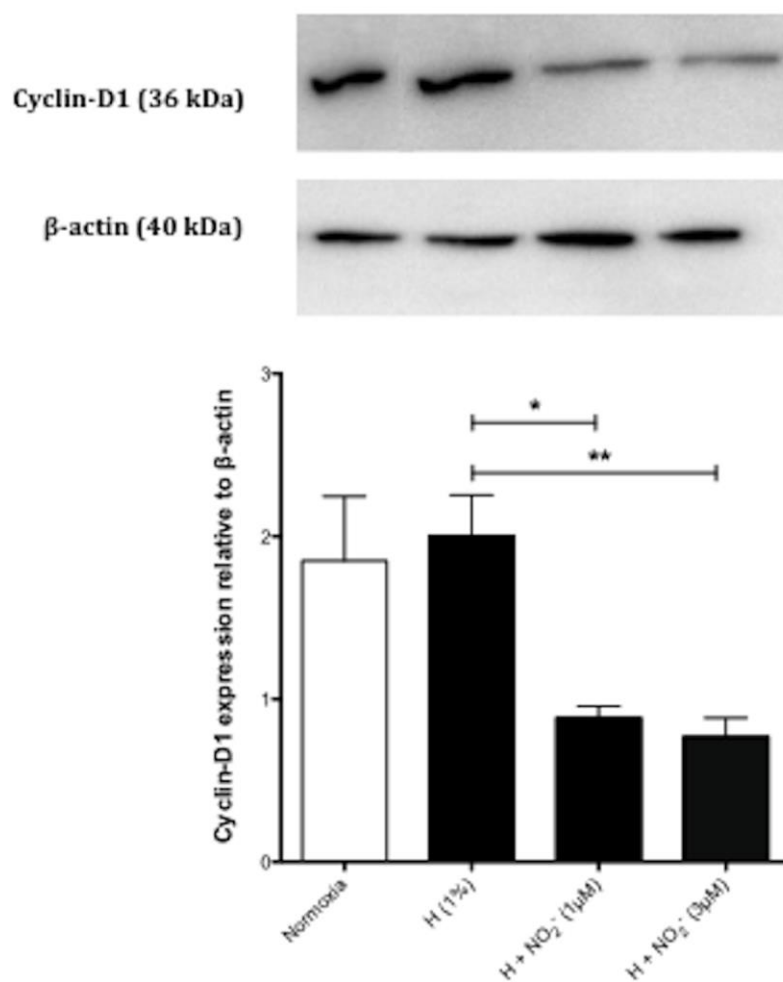
### 5.4.2 HmCASM

Nitrite increased p21 protein levels in a concentration-dependent fashion under 1% hypoxia with a significant increase in p21 levels shown with 3 $\mu$ M and 1 $\mu$ M nitrite treatment compared to control conditions (Figure 5.28). This increase in p21 was associated with a decrease in cyclin D1 levels (figure 5.29).



**Figure 5.28 Effect of nitrite (1 and 3  $\mu$ M) on p21 expression in HmCASCs.**

Nitrite and the effect on p21 expression in human coronary artery smooth muscle cells (HmCASC) under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means  $\pm$ SEM of n=5 independent experiments. Statistical significance shown as \*\* P<0.01 and \*P<0.05 comparing hypoxia with hypoxia + nitrite (3 $\mu$ M or 1 $\mu$ M), using 1-way ANOVA, with Bonferroni's multiple comparison test. H=Hypoxia.



**Figure 5.29 Effect of nitrite (1 and 3 μM) on Cyclin D1 expression in HmCASCs.**

Nitrite and the effect on Cyclin D1 expression in human coronary artery smooth muscle cells (HmCASC) under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means ±SEM of n=5 independent experiments. Statistical significance shown as \*\* P<0.01 and \*P<0.05 comparing hypoxia with hypoxia + nitrite (3μM or 1μM), using 1-way ANOVA, with Bonferroni's multiple comparison test. H=Hypoxia.

## **5.5 Expression of nitrite reductases**

### **5.5.1 E-NOS/P-eNOS/XOR expression in HCAECs**

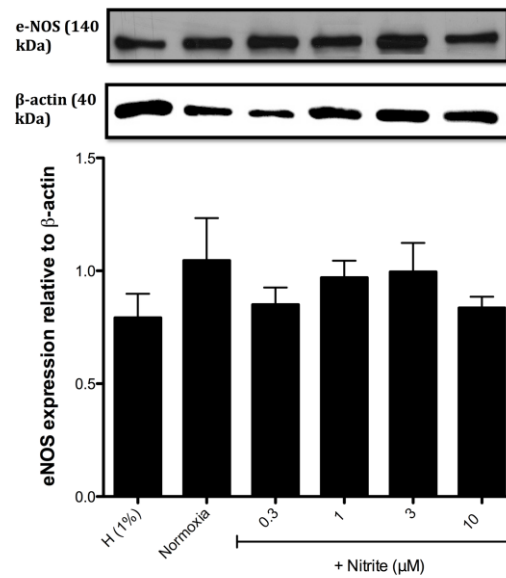
The expression of eNOS appeared to be decreased under hypoxic conditions however did not reach statistical significance. The addition of nitrite (0.3 $\mu$ M-10 $\mu$ M) did not alter eNOS expression (Figure 5.30A). Similar trends were seen with P-eNOS levels although again no statistically significant differences were seen (Figure 5.30B). The expression of XOR was greatest under normoxic conditions and lowest under hypoxic conditions, again nitrite increased XOR expression in comparison to hypoxia, although this trend was not significant (Figure 5.31)

### **5.5.2 XOR expression in HmCASM C**

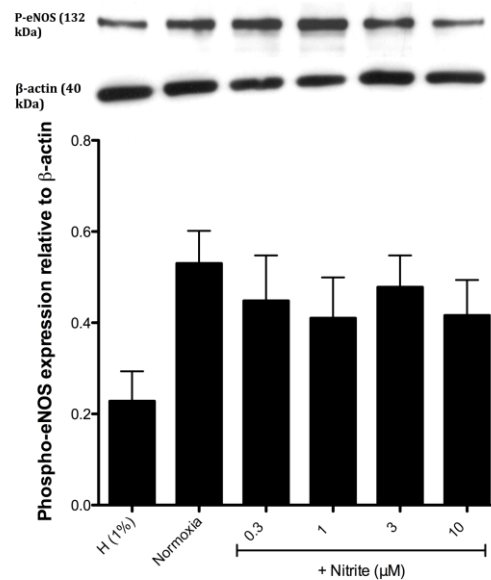
XO expression was the lowest under hypoxic conditions and highest with normoxia. The addition of nitrite increased the expression of XO compared to hypoxia alone, however, this trend was not significant (Figure 5.32).



A

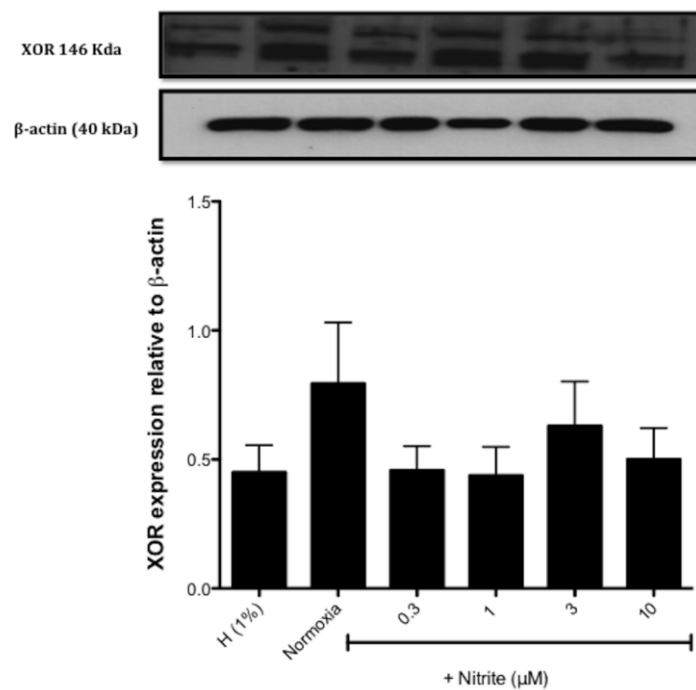


B



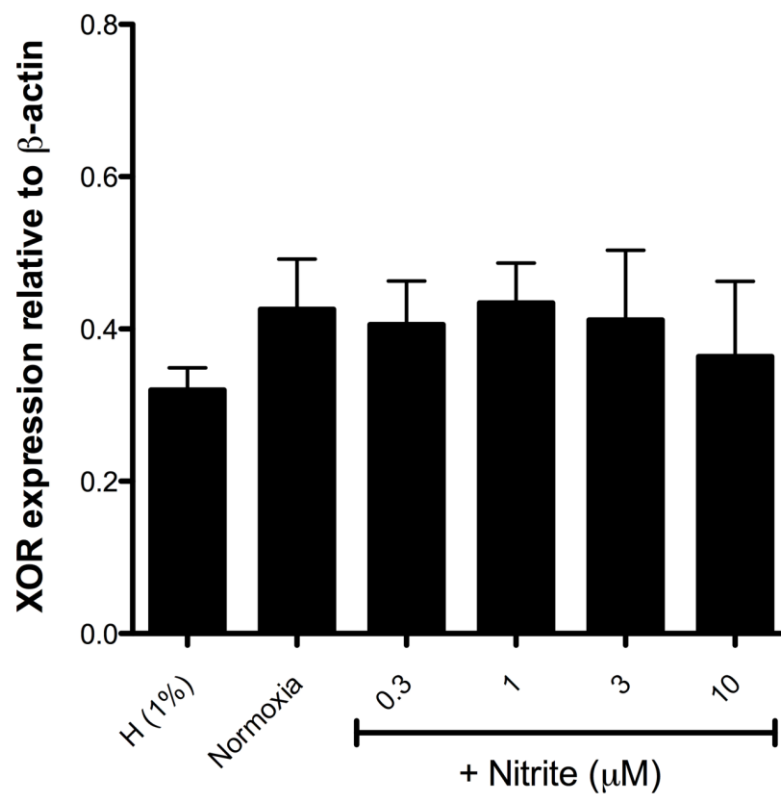
**Figure 5.30 Effect of nitrite (0.3-10  $\mu$ M) on nitrite reductases in HCAECs.**

Nitrite and the effect on nitrite reductase (e-NOS and phospho-ENOS) expression in human coronary artery endothelial cells (HCAEC) treated under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Panel A shows e-NOS expression with panel B showing phospho-ENOS. Data represent means  $\pm$ SEM of n=9 independent experiments for eNOS and n=6 independent experiments for phospho-ENOS. Statistical significance performed using 1-way ANOVA, with Bonferroni's multiple comparison test. H=Hypoxia.



**Figure 5.31 Effect of nitrite (0.3-10 μM) on XOR expression in HCAECs.**

Nitrite and the effect on XOR expression in human coronary artery endothelial cells (HCAEC) treated under 1% hypoxia (1% O<sub>2</sub>, 5% CO<sub>2</sub> and 37°C). Data represent means ±SEM of n=5 independent experiments. Statistical significance performed using 1-way ANOVA, with Bonferroni's multiple comparison test. H=Hypoxia, XOR=Xanthine oxidoreductase.



**Figure 5.32 Effect of nitrite (0.3-10  $\mu$ M) on XOR expression in HmCASM.**

Nitrite and the effect on XOR expression in human coronary artery smooth muscle cells (HmCASM) treated under 1% hypoxia (1%  $O_2$ , 5%  $CO_2$  and 37°C). Data represent means  $\pm$ SEM of n=5 independent experiments. Statistical significance performed using 1-way ANOVA, with Bonferroni's multiple comparison test. H=Hypoxia. XOR=Xanthine oxidoreductase,

## 5.6 Summary

1. Nitrite had no effect on the proliferation or viability of HCAEC/HUVEC or HmCASMCMC under normoxic conditions.
2. Hypoxia (5 and 1%) attenuated the proliferation and viability of both HUVECs and HCAECs compared to normoxia, associated with an increase in apoptosis.
3. Treatment of HUVECS and HCAECs with nitrite caused a concentration-dependent increase in endothelial cell number and viability in hypoxia with a peak effect occurring at 3 $\mu$ M in both cell types. The improvement in viability was associated with a reduction in apoptosis.
4. In contrast, whilst hypoxia also caused a decrease in HmCASMCMC numbers this effect was not associated with an increase in apoptosis. Moreover, the addition of nitrite caused a concentration-dependent decrease in cell growth with a peak effect occurring at 1 $\mu$ M vs normoxia. This decrease in cell number was not associated with a decrease in cell viability.
5. The effects of nitrite upon cell number and viability were attenuated by L-NMMA and allopurinol in endothelial cells and by allopurinol in HmCASMCMCs.
6. The sGC inhibitor ODQ attenuated the effects of nitrite on both HCAEC and HCASMCMC number and viability and these effects were associated with changes in cGMP.
7. Levels of cellular cGMP were significantly elevated in HCAECs and HmCASMCMCs treated by nitrite under 1% hypoxia compared to hypoxia alone, with levels of cGMP reduced by the addition of ODQ.

8. Nitrite (3 $\mu$ M and 1 $\mu$ M) resulted in enhanced endothelial cell proliferation and migration with reduced wound surface area compared to control using the scratch assay. This contrasted to HmCASCs where decreased cell migration and proliferation was seen with nitrite treatment (3 $\mu$ M and 1 $\mu$ M).
9. Levels of p21 protein expression were increased in nitrite (3 $\mu$ M and 1 $\mu$ M) treated HmCASC with a contrasting decrease in levels seen in HCAECs. In HmCASCs an associated decrease in cyclin D1 protein expression was seen with the elevation in p21 expression.
10. No difference in the expression of nitrite reductases (e-NOS, p-ENOS or XOR in endothelial cells or XOR in smooth muscle cells) were seen with nitrite treatment in cells treated under hypoxia.
11. I/R experiments demonstrated that reperfusion with nitrite (3 $\mu$ M and 1 $\mu$ M) following 0.1% O<sub>2</sub> (24h) resulted in a concentration dependent improvement in HCAEC growth and viability compared to reperfusion at all time points (P<0.01).
12. In HmCASCs reperfusion with nitrite (3 $\mu$ M and 1 $\mu$ M) resulted in a concentration dependent decrease in HmCASC growth with no detrimental effect on cell viability.

# **Chapter 6.0**

## **Discussion**

## 6.1 Introduction

This thesis has examined the use of intra-coronary nitrite as a strategy to reduce myocardial I/R injury in patients undergoing primary PCI for AMI. Since myocardial I/R injury was first described, there is yet to be a successfully translated technique adopted to routine clinical practice for its treatment. Few therapies have replicated the protection shown in the animal setting. Coronary heart disease still represents a huge burden of morbidity and mortality (see Chapter 1) and there is no doubt that further improvement in treatment is needed. Novel strategies are required and 'cardioprotection' has long been regarded as one method, which may be successful in accomplishing this.

This thesis suggests that intra-coronary nitrite infusion appears to be safe and tolerable in patients undergoing primary PCI for AMI. Furthermore in patients with TIMI flow  $\leq 1$  at time of drug delivery it may reduce reperfusion injury, and subsequent infarct size. This thesis proposes that this effect is achieved through several mechanisms with effects demonstrated on both platelet reactivity, and inflammation. It appears that nitrite has selective effects on the production of neutrophil specific cytokines, and subsequent neutrophil activation, reducing the inflammatory component of I/R injury. The data presented also proposes that nitrite may have potential beneficial effects on outcomes following stent implantation, both inhibiting smooth muscle proliferation and therefore preventing in-stent restenosis and also promoting re-endotheliasation of drug-eluting stents minimising the potential for stent thrombosis.

Three key aims were set out by this thesis, which will be discussed in detail during this discussion.

## **6.2 Intra-coronary nitrite is safe, tolerable and may reduce infarct size during primary PCI in patients with AMI.**

In chapter 3, it was demonstrated in a proof-of-concept study that the intra-coronary administration of nitrite at the time of reperfusion in patients with AMI is safe but was not associated with a reduction in infarct size assessed by cardiac enzymes, compared to that seen with placebo. However, there was a trend to a greater myocardial salvage index with an 18% increase in the nitrite group compared to placebo (this was on the boundaries of conventional statistical significance ( $p=0.051$ )), and significant reductions in MACE at both 6 months and 1 year. This weight of evidence could suggest an effect associated with nitrite treatment despite no reduction in cardiac enzyme assessed infarct size.

In a single sub-group analysis of patients with TIMI flow  $\leq 1$ , at the time of primary PCI, treatment with nitrite was associated with a 20% reduction in infarct size compared to placebo as assessed by cardiac biomarkers (AUC for CK). This observation was supported by the CMR analyses in this sub-group demonstrating a reduction of infarct size in the region of 25%, a reduction in microvascular obstruction by 48% and an association with a significantly greater myocardial salvage index. This data is therefore suggestive of an effect with multiple supporting modalities however it is important to note that although this was a single subgroup analysis it was not pre-specified. No evidence of a



difference in infarct size was seen between nitrite and placebo in patients with TIMI flow >1.

#### **6.2.1 Ischaemia time and AAR are important confounding variables**

Important determinants of infarct size after primary PCI include the duration of ischaemia (Christian et al., 1992) and the AAR (Reimer et al., 1977), both of which were confounding variables in this study. Despite the use of best practice through randomization and double-blinding nitrite-treated patients had a longer mean ischaemia time compared to the placebo treated group, which is known to adversely effect potential myocardial salvage and infarct size (Keeley and Hillis, 2007; Armstrong et al., 2009). In this study ischaemia time was directly correlated to infarct size measured by either CK or troponin T confirming the relationship in this cohort. It is therefore possible that the absence of a significant effect of nitrite in all-comers has been influenced by this variation. Importantly, in the single sub-group analysis based on TIMI flow, where a beneficial effect of nitrite was seen there were no differences in ischaemia time. The mean ischaemia time in the whole cohort (~189 minutes) reflects well when compared to other studies assessing potential adjunctive cardioprotective strategies e.g. cyclosporine (Piot et al., 2008) and postconditioning (Staat et al., 2005) (Thuny et al., 2012) with values ranging from an average of 331-252 mins. It is known that myocardial reperfusion accrues the most benefit in terms of myocardial salvage in patients presenting within 3 hours of chest pain onset (Gersh et al., 2005). Whether infarct size reduction with a study intervention is greater in patients presenting early (within 3 h) or later (3 h and beyond) is not clear however two

clinical studies have reported greater benefit (myocardial salvage) with potential cardioprotective agents adenosine (Kloner et al., 2006) or exenatide (Lonborg et al., 2012a) in patients presenting within 2–3 h of chest pain onset, suggesting that the former may be true. However laboratory studies suggest the relative importance of different mechanisms of I/R injury may depend on the duration of ischaemia, with mitochondrial permeability transition playing a more prominent role after prolonged ischaemia (60 minutes) (Ruiz-Meana et al., 2011). The median ischaemia time of 189 minutes in our cohort or 181 minutes in the TIMI <1 subgroup reflects well compared to this 3-hour mark. Importantly, the ischaemia time in this study likely underlies the smaller infarct sizes seen when compared to other myocardial injury studies (Piot et al., 2008) (Staat et al., 2005).

The size of the AAR is a major determinant of the final infarct size (Reimer et al., 1977). Previous clinical cardioprotection studies in STEMI patients suggest that the patients most likely to benefit from a study intervention administered as an adjunct to myocardial reperfusion are those presenting with a large AAR (>30% of the LV) (Staat et al., 2005; Piot et al., 2008; Botker et al., 2010). Indeed recently recommendations suggest that potential cardioprotective agents are tested in patients with AAR >30% (Hausenloy et al., 2013). The mean AAR in the whole of the study cohort was 33% assessed by CMR and just under 30% by either angiographic score which compares well to these recommendations.

Assessing the AAR is particularly important as the size of the AAR can vary greatly from patient to patient (from 10 to 50% of the LV) depending on which coronary artery is involved (LAD, RCA, or Cx) and where along the vessel the

occlusion has occurred (proximal, mid-vessel, or distal) (Ortiz-Perez et al., 2007). In this study the mean AAR assessed by either CMR or angiographic scores whilst not statistically different displayed a trend to be larger in the nitrite-treated relative to the placebo group, in the whole cohort with increases ranging from 5% to 14% depending on the method of assessment. There are recognised limitations to each of the methods that were used to assess AAR (Botker et al., 2012), however, the data showed significant positive correlations between them with consistently larger scores in the nitrite group. This could confound the results with theoretically larger infarcts in the nitrite group in view of the larger AARs. The myocardial salvage index, which corrects for the AAR was greater in the whole cohort (although not significantly so) in the nitrite group, which further suggests that the larger AARs and longer ischaemia times are likely to be significant confounding factors.

### **6.2.2 Lack of benefit in patients with TIMI flow >1 is not unexpected**

Despite the important confounders of AAR and ischaemia time, in patients with significant coronary flow (TIMI flow >1) prior to nitrite infusion a lack of benefit of nitrite is not entirely unexpected. Pre-clinical models have shown beneficial effects of nitrite on I/R injury that occur with nitrite administered prior to reperfusion (TIMI flow  $\leq 1$ ). The rationale for evaluating the ability of nitrite to reduce infarct size in patients with ongoing AMI was based on extensive pre-clinical evidence demonstrating the cytoprotective properties of nitrite in models of AMI (Webb et al., 2004; Duranski MR et al., 2005; Shiva et al., 2007b; Baker JE et al., 2007) and in other organs including the liver (Duranski MR et al., 2005),

kidney (Tripathara et al., 2007) and brain (Pluta et al., 2005). In all of these studies the beneficial effects were evident with application of nitrite into or on the ischaemic organ with the culprit vessel occluded at time of drug delivery i.e. zero flow. Whilst nitrite bioactivation to NO within the circulation does occur under physiological conditions in humans (Cosby et al., 2003) (Maher et al., 2008), this phenomenon is enhanced with decreasing oxygen tension and this enhancement underlies its improved bioactivity under hypoxic/ischaemic conditions (Gladwin et al., 2005).

If a cardioprotective strategy needs to be administered prior to or at the immediate onset of myocardial reperfusion, it could be argued that there is little rationale for recruiting patients who do not have a fully occluded coronary artery (TIMI >1) at presentation. However standard care pathways for STEMI patients presenting to hospitals for primary PCI include early and efficient administration of therapies especially anti-platelet and anti-thrombotic drugs, as in the present study. As a consequence it has been suggested that >40% of patients will have spontaneously reperfused in the infarct related territory resulting in significant coronary flow (TIMI flow >1) within the culprit coronary artery prior to the procedure (Botker et al., 2010; Lonborg et al., 2010). Assessing whether nitrite would benefit or indeed cause no harm to a representative group of patients including those with TIMI flow >1 is important, particularly when one considers that translation of cardioprotective interventions can only legitimately occur if there is no delay to revascularization. Thus, a strategy that might be standardly administered to all patients with no need for time spent on assessing suitability or not is the ideal scenario and underlies the rationale for the design of the trial.

However, the low numbers of patients with TIMI flow  $>1$  (10%), in our cohort prevent drawing any reliable conclusions with respect to the effects of nitrite in these circumstances. In contrast the findings do suggest that, as expected, for nitrite to be most effective against myocardial I/R injury administration should occur while the culprit artery is occluded and prior to myocardial reperfusion, as in the TIMI flow  $\leq 1$ . This is similar to other cardioprotective agents such as remote ischaemic conditioning where patients presenting with an occluded culprit artery accrued the most benefit in terms of infarct size reduction (Botker et al., 2010), although occasional studies have shown an overall benefit in all comers irrespective of TIMI flow (Botker et al 2010). In view of the numbers of patients with TIMI flow  $>1$  being low i.e. nine in total with only four in the nitrite group, further appropriately powered studies assessing safety in patients with TIMI flow  $>1$  are essential to determine the generalised safety of intra-coronary nitrite administration in patients presenting with AMI.

### **6.2.3 Intracoronary nitrite appears safe with no demonstrated acute adverse effects**

The intra-coronary administration of nitrite exerted no adverse effects in the cohort as a whole or in either of the TIMI flow  $\leq 1$  or  $>1$  subgroups. Specifically, blood pressure was assessed due to the known vasodilator (Cosby et al., 2003; Frenneaux et al 2007) and blood pressure lowering effects (Dejam et al., 2007; Pluta et al., 2011) of raised circulating nitrite levels. In this study circulating nitrite levels were significantly elevated in the patients who received intra-coronary nitrite but not in the patients who received placebo. The data

demonstrate that whilst blood pressure did drop in some patients this was equally evident in both arms. Bradycardia and hypotension are a common feature of reperfusing occluded coronary arteries (Bezold-Jarisch reflex) particularly the right coronary artery. Studies have shown that 65% of reperfusing right coronary artery occlusions (Esente et al., 1983) can result in this phenomenon and it is a well-established feature (Mark, 1983). A large proportion of the effect occurred in patients with occluded right coronary artery with accompanying bradycardia (70%). Similarly levels of methaemoglobin were monitored due to the known interaction of nitrite with oxyhaemoglobin to generate methaemoglobin and, in particular, the elevated levels of this measure with systemic nitrite infusions (Pluta et al., 2011). Levels of methaemoglobin were comparable between the two treatment arms and at levels that are not symptomatic. In this study, nitrite administration was not associated with either lowering of blood pressure or methaemoglobinaemia, the two main potential concerns with this form of treatment.

#### **6.2.4 Comparison with other studies assessing nitrite in AMI**

The results of this study demonstrating no evidence of a benefit of intra-coronary nitrite in the whole cohort is consistent with recent emerging data from an intravenous nitrite in AMI study which also showed no reduction in infarct size (Siddiqi et al., 2014). An important difference with this study is Siddiqi and colleagues only recruited patients with TIMI  $\leq 1$  flow and not all-comers. They recruited a total of 229 patients presenting with AMI who were randomized to receive either an intravenous infusion of 70 $\mu$ mol sodium nitrite (n = 118) or

matching placebo (n = 111) over 5 min immediately before primary PCI. This dose was chosen assuming a mean body weight of 70 kg, corresponding to 1  $\mu\text{mol/kg}$ . The primary endpoint of myocardial infarct size (assessed at 6–8 days by CMR) did not differ between nitrite and placebo groups with a median infarct size of 22% in the nitrite and 20% in the placebo groups [difference  $-0.7\%$  (95% CI:  $-2.2, +0.7$ ;  $P = 0.30$ )]. After adjustment for AAR, diabetes, or each recruitment centre (patients were recruited at 4 different centres) there was still no difference between the groups (effect size  $-0.7\%$  95% CI:  $-2.2\%, +0.7\%$ ;  $P = 0.34$ ). There were also no significant differences in any of the secondary endpoints, including plasma troponin I or CK AUC (72-hour), LV volumes or LVEF measured at 6–8 days and infarct size at 6 months. This study would suggest that the intravenous route of nitrite administration does not result in decreased infarct size or improve outcomes after primary PCI with the secondary endpoints and subgroup analyses all consistent with their negative primary endpoint.

The study by Siddiqi and colleagues was based on a protocol employed in dogs (Gonzalez et al., 2008) in which a 5 min infusion of sodium nitrite administered immediately prior to opening of the infarct-related artery was shown to substantially reduce infarct size. The study by Gonzalez and colleagues showed similar reductions in infarct sizes with either a 5 or 60 min infusion prior to reperfusion suggesting that increasing the duration of the infusion is unlikely to be of benefit. With respect to dose, pre-clinical evidence indicates that nitrite is cytoprotective against I/R injury only when given at concentrations (2.5-10  $\mu\text{mol}$ ) that far exceed physiological (0.1-0.4  $\mu\text{mol/L}$ ) levels of circulating nitrite levels. In the study by Siddiqi and colleagues circulating nitrite levels achieved

with a 5 minute infusion were elevated to 1.4 $\mu$ mol/L, which is lower than that seen in dogs where the same dosing regime led to circulating levels of 5  $\mu$ mol/ (Gonzalez et al 2008). However the levels achieved were 7.8-fold higher than in the placebo group and consistent with levels achieved in a previous murine periconditioning study that demonstrated at least some molecular effect of nitrite in mice at such concentrations (Duranski MR et al., 2005).

It is possible that the lack of benefit in the Siddiqi study simply reflects the complexities of translation of animal studies into the clinical setting. However the lack of benefit seen could be due to an insufficient elevation of nitrite levels achieved in the target myocardium to induce cardioprotection, and that achieving this is not possible via the intravenous route. Indeed raising circulating levels of nitrite to those shown effective in most pre-clinical studies is likely to be associated with adverse effects such as hypotension and methaemoglobinaemia as demonstrated in healthy volunteers (Pluta et al 2011). We speculate that the lack of adverse effect seen in our study despite the use of high (supra-physiological) levels of nitrite given intra-coronary likely reflects the advantage of intra-coronary nitrite administration. This route achieves high local concentrations (2.5-10 $\mu$ M) within the myocardium only, minimising the potential systemic side-effects which may occur with systemic nitrite administration. Indeed although levels of systemic nitrite were elevated in the study patients who received intra-coronary nitrite indicating a degree of systemic absorption, levels achieved were approximately 0.5 $\mu$ M/L, only marginally elevated compared to physiological levels, which would be in keeping with the low rates of side-effects seen in the study.



The intra-coronary route has other potential advantages. If agents are to be delivered at reperfusion i.e. following angiography to ensure TIMI flow  $\leq 1$  the intra-coronary route will not delay reperfusion. This is compared to other therapies such as cyclosporine and exenatide where intra-venous administration may result in both greater side-effects and a delay in reperfusion whilst administered. Mechanical therapies associated with re-intervention such as ischaemic postconditioning, where repeat balloon inflation is conducted after the opening of the culprit vessel, carry a potential risk of damaging the vessel or inducing peripheral embolization.

#### **6.2.5 Improvement in clinical outcomes after intracoronary nitrite**

This thesis demonstrated a reduction of infarct size of  $\sim 19\text{-}24\%$  evident in response to nitrite treatment in those patients with TIMI flow  $\leq 1$ . This value is comparable to the reductions of infarct size estimated to occur with other cardioprotective agents (Piot et al., 2008; Mewton et al., 2010; Thibault et al., 2008) tested in similar patient populations (TIMI  $\leq 1$  flow). Whether such a difference might result in an improvement of outcome long-term is uncertain, with none of the studies mentioned above having clinical endpoints such as MACE. Infarct size has been shown to be associated with a reduction in mortality but the majority of studies testing cardioprotective agents have either not shown or not tested for this effect. However in the study described in this thesis, nitrite treatment in the whole cohort was associated with a reduction in MACE at both 6 months and 1 year, which would correspond to the decreased myocardial infarct size. It is important to recognise the low number of total events reported and

acknowledge that larger studies with MACE as primary outcome are needed to verify this. However, in support of this data is very recent evidence demonstrating that cardioprotective strategies such as post-conditioning post-PCI do positively impact on long term outcome measures (Sloth et al., 2014) and it should be noted that at least in one study exploring the value of post-conditioning strategies, reductions in cardiac biomarkers release was associated with significant improvements in cardiac function (left ventricular ejection fraction) at 1 year (Thibault et al., 2008). To date few studies have been sufficiently powered to determine whether cardioprotective strategies might improve outcome and although in this present study a reduction in MACE was evident in patient's 1-year post-nitrite treatment, the study was not powered for MACE events. This data warrants further investigation in larger phase III clinical trials powered by reduction in MACE.

### **6.3 Mechanisms by which nitrite reduces reperfusion injury, focusing specifically on inflammation and platelet activity**

The second key aim of this thesis was to investigate the potential mechanisms by which intra-coronary nitrite reduces I/R injury and subsequent infarct size. It was clear from pre-clinical models that the beneficial effects of nitrite are due to its conversion to NO, as evidenced by the complete block of nitrite-induced protection by NO scavengers (Webb et al., 2004; Duranski MR et al., 2005). NO itself exerts a number of actions (Moncada, Higgs 1993) that are thought to be cardioprotective (Johnson 1991). In particular NO plays an important role in modulating many aspects of the inflammatory response. (Kubes et al., 1991; Ahluwalia et al., 2004; dPalazzo et al., 1998) and in reducing platelet aggregation and reactivity (Davi, Patrono, 2007; Radomski et al, 1987; Radomski et al, 1990). Potential anti-platelet and anti-inflammatory effects of nitrite when given intra-coronary during primary PCI were therefore assessed.

#### **6.3.1 Inflammatory response following primary PCI for AMI**

AMI evokes an intense inflammatory response both systemically and within the infarcted myocardium with a release of pro-inflammatory cytokines as discussed in detail in chapter 1, but including TNF- $\alpha$ , CXCL-1 and CXCL-5. Patients with large infarcts often have elevation of body temperature, WBC count, complement, CRP, and other inflammatory markers compatible with a systemic inflammatory response syndrome reaction (Balbay et al., 2001; Modica et al., 2009). This release of inflammatory cytokines has been shown in patients after primary PCI

for AMI but not in control patients (Neumann et al., 1995). The scale of this response reflects the size of the infarction (Van Diepen et al., 2013) with excessive early inflammation after AMI associated with adverse left ventricular remodeling and poor outcomes (Anzai et al., 1997; Suleiman et al., 2006) with the inflammatory response known to be a key component of reperfusion injury (Yellon and Hausenloy, 2007). Modulating and reducing this inflammatory response is therefore likely key to improving outcomes after primary PCI for AMI.

A marker of this early inflammation is enhanced synthesis of CRP (Frangogiannis et al., 2002). We showed that hs-CRP increased in the first few hours after reperfusion, peaking at 24 hours, which is consistent with the current literature (Uehara et al., 2003). CRP release is stimulated by upstream cytokines (such as IL-6, with which CRP keeps a high correlation in the release pattern after AMI (Gabriel et al., 2004)), and reflects the degree of the inflammatory response (Nian et al., 2004; Orn et al., 2009; Suleiman et al., 2006). Indeed, the extensive presence in the context of AMI, and its known participation in complement activation mean that CRP has been postulated as an actual mediator of intense myocardial damage (Nian et al., 2004; Orn et al., 2009). However, irrespective of whether CRP is a marker of the post AMI inflammatory response or a contributory component, levels of hs-CRP were significantly reduced after reperfusion in the nitrite treated group compared to placebo suggesting a reduced inflammatory response in this group. Hs-CRP levels were shown to correlate with infarct size and therefore rather than this reduction being due to a direct effect of nitrite it is likely to simply reflect the smaller infarcts seen in the nitrite group. Indeed, such a view is substantiated by associated reductions in

other markers of inflammation such as WBC and MCP-1. Importantly CRP determination in the first 2 days after primary PCI has been found to show significant predictive value for clinical outcomes, (Ohlmann et al., 2006) therefore irrespective of aetiology, lower levels of hs-CRP in the nitrite treated group would suggest a benefit.

### **6.3.2 Reduction in general markers of inflammation with nitrite treatment**

Aside from CRP, another general marker of inflammation linked with damage after AMI is an increase in the peripheral white blood cell (WBC) count, which correlates with outcomes after AMI (Furman et al., 1996). A large-scale study with 153,213 patients aged 65 years or older with AMI demonstrated that a higher WBC count within 24 h of admission was associated with higher mortality within 30 days (Barron et al., 2001). Studies have also demonstrated that elevated WBC, specifically neutrophils, either pre or post primary PCI is associated with increased infarct size and impaired LV function (Sabatine et al., 2002; Shinozaki et al., 2005; Chia et al., 2009). In fact data exists to suggest that WBC count post procedure is a better predictor of outcome than at baseline (Shinozaki et al., 2005) and that WBC count after AMI not only decreases within hours but is also related to the success of reperfusion therapy as reflected by TIMI flow and MBG (Smit et al., 2006; Mariani et al., 2006). This would indicate that the rise in neutrophils after AMI is dependent on microvascular function and may be used as a non-invasive marker of failed or incomplete myocardial reperfusion or degree of I/R injury. Our data is consistent with this, demonstrating a decline in WBC count early (4 hours) after reperfusion and that

lower levels of WBC found in the nitrite patients post procedure appear to be associated with both improved ST segment resolution (a similar measure to MBG), smaller infarct size and improved outcomes. This adds further indirect evidence of reduced injury in the nitrite group but also suggests that a reduction of the systemic and perhaps local inflammatory response in the nitrite group could be responsible for this benefit.

### **6.3.3 Nitrite treatment was associated with decreased neutrophil specific chemokine expression**

In myocardial I/R injury, inflammatory cell recruitment in the myocardium is dominated by early neutrophil recruitment, which induces tissue damage through a variety of mechanisms as discussed in chapter 1 (for review see Vinten-Johansen, 2004). Thus, the selective inhibition of neutrophil chemoattractant bioactivity might prevent myocardial injury during the early phases of reperfusion. Key to this recruitment are neutrophil specific chemokines (such as CXCL-5 and CXCL-1) that have been shown to be up-regulated in models of experimental AMI and promote activation of neutrophils and chemotaxis to the site of tissue injury (Frangogiannis and Entman, 2005; Frangogiannis, 2004; Kobayashi, 2008). CXCL-1 (also known as human growth-related oncogene-  $\alpha$  and KC) was first described as a growth factor for human melanoma cell lines, binds to CXCR2, and belongs to the IL-8 family (Frevert et al., 1995; Bozic et al., 1995). The release of CXCL-1 and CXCL-5 are regulated by pro-inflammatory cytokines (TNF- $\alpha$ ) and transcription factors such as NF- $\kappa$ B (Chandrasekar et al., 2001). Increased plasma CXCL-1 levels and induction of CXCL-1 expression has been

detected in both rat (Chandrasekar et al., 2001; Getting et al., 2004), and mouse (Mersmann et al., 2010) models of myocardial I/R injury and has also been observed after I/R in murine kidneys and skeletal muscle (Molls et al., 2006). Plasma CXCL-5 levels have also been shown to be elevated in rat models of myocardial I/R injury (Chandrasekar et al. 2001). In a model of renal I/R, neutralizing CXCL-1 antibodies decreased mortality and improved renal function (Miura et al., 2001). This is in accordance with smaller infarcts seen after myocardial I/R in mice lacking CXCR2 (Tarzami et al., 2003) and importantly a reduction in reperfusion injury seen in a mouse model of AMI following a single administration of the CXC Chemokine-Binding Protein, Evasin-3 which prevented CXCL1 induced neutrophil recruitment and reactive oxygen species production in the myocardium (Montecucco et al., 2010). Therefore, the reduced levels of CXCL-1 seen in the nitrite treated patients may result in reduced neutrophil activation, which is likely to be key to any reduction in infarct size seen in this group. Importantly this reduction in neutrophil activation was demonstrated by the lower levels of CD11b expression seen in the nitrite treated patients. The levels of CXCL-1 and CXCL-5 were both shown to be related to infarct size and be reduced in the nitrite treated patients, implying that by preventing cell death and reducing infarct size the subsequent inflammatory response is less.

It is also possible that nitrite-derived NO is directly affecting CXCL-1 generation. Release of CXCL-1 from human endothelial cells (HUVECs) in response to TNF- $\alpha$  has been shown to be dependent upon p38 MAPK and PI-3kinase-mediated secretory processes (Lo et al., 2014). Since NO is known to effect various intracellular signaling molecules including MAPK and JAK (Stamler et al., 2001) it

is possible that nitrite-derived NO effects cytokine release during I/R injury. Additionally cytokine generation in the myocardium is regulated at the transcriptional level by NF- $\kappa$ B (activated by cytokines themselves or by ROS) (Chandrasekar and Freeman, 1997; Fan et al., 2002) and at high concentrations NO inhibits NF- $\kappa$ B activation (Cooke and Losordo, 2002).

MCP-1 expression has been shown to be markedly but transiently induced in infarcted hearts (Dewald et al 2004, Frangiogiannis 2007) with it appearing to critically regulate the healing response (Gonzalez-Quesada, Frangiogiannis 2009). Studies using post ischaemic cardiac lymph collected during canine reperfused infarction suggest that MCP-1 may be a major factor responsible for mononuclear cell recruitment into the ischaemic myocardium during the first five hours of reperfusion (Birdsall et al 1997). In addition, in a rat model of experimental myocardial infarction, administration of a neutralizing antibody to MCP-1 significantly reduced infarct size decreasing adhesion molecule expression and macrophage infiltration (Ono et al 1999). Beyond these actions, MCP-1 has important effects on infarct healing mediated through its direct angiogenic effects on the vascular endothelium, or by modulation of fibroblast phenotype and activity (Gharaee-Kermani M et al 1996). Prolonged elevation of plasma MCP-1 therefore may identify patients who mount a more intense cardiac inflammatory reaction following a coronary event, or show impaired resolution and defective containment of the post-infarction inflammatory response (Gonzalez-Quesada, Frangiogiannis 2009). Therefore the higher levels of MCP-1 seen in the placebo group are consistent with the overall increased inflammatory response compared to nitrite treated patients. Additionally MCP-1 levels have been demonstrated to



be independently associated with the development of no-reflow and 3-year mortality in patients with ST-segment elevation myocardial infarction undergoing primary PCI (Buyukkaya et al 2103), a finding consistent with the lower MACE rates and accompanying the lower peak MCP-1 levels seen in the nitrite group.

#### **6.3.4 NOx levels after primary PCI are a reflection of iNOS activity**

Intuitively it may have been expected that plasma nitrite levels were higher in those patients who received intra-coronary nitrite, however whilst acutely nitrite levels were elevated at 30 minutes post-reperfusion by the intervention, over time both nitrite and nitrate levels decreased with a greater reduction in the nitrite treated patients. This elevation in NOx levels in the placebo group is likely to reflect the greater systemic inflammatory response seen in these patients as a consequence of the greater infarct size. This would result in a greater degree of iNOS expression and subsequent elevated levels of NOx. This data is supported by the higher levels of inflammatory mediators (hs-CRP and MCP-1) seen in the placebo group and significant correlations between infarct size, levels of inflammatory mediators and cGMP levels.

Evidence of iNOS induction in the heart following AMI comes mainly from pre-clinical studies, however levels have also been shown to be elevated in human myocardium (Wildhirt et al., 1995a). Wildhirt and colleagues identified the presence of iNOS in hearts subjected to MI in a rabbit coronary ligation model, demonstrating at least a two-fold increase in iNOS expression in the infarcted

region, mainly localized to the infiltrating macrophages (Wildhirt et al., 1995b). However, whether iNOS is harmful or protective during I/R injury after AMI has been much debated with apparent discrepancy among previous studies, some of which have concluded that iNOS is beneficial (Bolli et al., 1997; Das et al., 2005; Li et al., 2006; Heger et al., 2002), and crucial to the effects of pre-conditioning, whereas others have found it to be detrimental (Feng et al., 2001; Gealekman et al., 2002; Sam et al., 2001; Liu et al., 2005). Reasons for these discrepancies are unclear however they may relate to the source of iNOS and levels of NO. A recent study using mouse models of myocardial infarction, demonstrated that selective deletion of iNOS from peripheral blood cells (with no change in myocardial iNOS content) reduces infarct size, suggesting that iNOS derived from peripheral blood cells is detrimental during myocardial I/R injury whereas myocardial iNOS is protective (Guo et al., 2012). If iNOS can be beneficial or detrimental depending on the cell type in which it is expressed this would explain the differences in the studies performed to date with results depending on the level of inflammatory cell activation and infiltration into the infarct, which may be related to model specific factors. This would fit with the data shown in this thesis, with high local (myocardial) NO levels beneficial during I/R injury associated with a reduction in infarct size whereas those patients with larger infarcts, greater cytokine release and greater inflammatory responses will have greater systemic iNOS activation, which is both detrimental and also might explain the higher NO<sub>x</sub> levels seen.

### **6.3.5 Reduced reperfusion injury demonstrated in nitrite treated patients**

A direct measurement of reperfusion injury in the study was provided by the CMR assessed microvascular obstruction (MVO) and the degree of ST-segment resolution on the ECG, both of which were found to be reduced in the nitrite treated TIMI <1 patients compared to placebo. MVO, a type of irreversible reperfusion injury is believed to be a consequence of factors such as distal embolisation, leucocyte migration and plugging and platelet aggregation (Lee and Tse, 2010). Importantly, MVO has been implicated in worse clinical outcomes due to poor myocardial perfusion despite epicardial coronary artery revascularization (Hombach et al., 2005). In this study, the incidence of MVO detected by the initial CMR scan in the whole CMR population decreased from 69.7% in placebo to 48.5%, in the nitrite treated group, whilst in the TIMI flow <1 subgroup it decreased from 72.4% to 37.0% respectively. It is worth noting that factors known to impact on MVO, such as the presence of comorbid conditions and the use of antiplatelet and anticoagulant therapy, were similar between the two treatment groups. It is known that infarct size is related to the degree of MVO (Iwakura et al., 2001) and this association may simply underlie the apparent decrease. However, whilst CMR-determined infarct size was reduced by 24%, MVO was reduced by 48% suggesting that factors other than the size of infarct are involved. These factors could include the reduced platelet reactivity that has been demonstrated in the nitrite treated patients or reduced leucocyte migration/plugging as a consequence of reduced neutrophil activation (decreased levels of neutrophil chemokines/activation markers).

Persistent ST elevation on the ECG despite normal epicardial blood flow is indicative of impaired tissue and microvascular perfusion and hallmarks of I/R injury (van't Hof et al., 1998; Santoro et al., 1998). It is associated with poor recovery of left ventricular function and increased mortality after primary PCI even with successful restoration of TIMI grade 3 flow in the infarct artery (Somitsu et al., 1997; Claeys et al., 1999). In the study cohort ST segment resolution of >70% was evident in 88.5% of both treatment groups. However when looking at TIMI  $\leq 1$  subgroup, 100% of the nitrite group and 84.8% of the placebo groups had ST segment resolution ( $p=0.02$ ). This observation provides further support for the view that nitrite treatment appeared to result in a decreased I/R injury in the nitrite-treated patients.

#### **6.3.6 Reduced platelet reactivity is a consequence of reduced inflammation**

In patients following primary PCI for STEMI, platelet reactivity was enhanced with elevated P-selectin expression that decreased over time, and with the decrease significantly enhanced in the nitrite compared to the placebo group. The response of platelets to activating stimuli (ADP and collagen) also decreased over time in the whole cohort but the decreases were substantially greater in the nitrite-treated patients. Although NO has well documented direct anti-platelet effects this reduction in platelet reactivity probably reflects the reduced infarct size and systemic inflammation that exist in the nitrite group, rather than a direct effect. Indeed post-hoc analyses show a direct correlation between platelet reactivity and infarct size supporting this hypothesis. However, irrespective of aetiology, lower platelet reactivity is likely to contribute to the reduced

complications evident at 6 months and 1 year after STEMI in the nitrite patients, with platelet reactivity at 6 months after STEMI linked to the recurrence of coronary events during this time period, even in the modern era of multiple antithrombotic therapy (Scalone et al., 2011).

Interestingly platelet reactivity was not the only measure shown to be reduced in the nitrite group at 6 months, there was also a reduction in the levels of circulating WCC, systemic hs-CRP/MCP-1 levels and reduced NT-pro-BNP all which may be all related to reduced levels of inflammation however 6 months after reperfusion and infarction there may be other explanations and a potential link with vascular function.

### **6.3.7 Improved vascular health at 6 months in nitrite treated patients**

Prospective epidemiological studies have shown that serum hs-CRP levels are a simple tool for detecting low-level systemic inflammation and are a strong predictor of the occurrence of cardiovascular events such as MI in apparently healthy subjects (Ridker et al., 1997; Ridker et al., 2000) and in patients with a recent coronary event (Ridker et al., 2005). This elevation in hs-CRP is believed to reflect inflammation in the vascular bed or an intensification of focal inflammatory processes that destabilize vulnerable plaques (Morrow and Braunwald, 2003). As discussed previously the acute inflammatory response associated with myocardial necrosis is associated with a significant elevation in hs-CRP levels but this elevation has been shown to subside within 4 to 6 weeks, with hs-CRP levels returning to baseline values (James et al., 2005; Argaud et al., 2005). At this time, elevated hs-CRP levels again reflect the degree of vascular inflammation and are predictive of future coronary events (Ridker et al., 2005). Therefore, importantly hs-CRP levels measured in patients following AMI, after levels return to normal (after 30 days), are predictive of further cardiovascular events. This is of particular relevance to this thesis as higher levels of hs-CRP were demonstrated at 6 months in patients treated with placebo compared to those that received nitrite. This is likely to reflect a persistently elevated level of inflammation with impaired vascular health and endothelial dysfunction in these patients compared to those that received nitrite therapy. Whether this is related to the reduction in infarct size seen acutely or another mechanism is unclear. However levels of hs-CRP have been shown to be related to measures of vascular and endothelial function (Huang et al., 2007) such as pulse wave velocity (Nagano

et al., 2005; Kim et al., 2007) and therefore these higher levels would support this impaired function. In our study we did not assess vascular function and these further studies measuring both endothelial function and vascular stiffness post PCI and nitrite treatment would be of value.

The hypothesis of impaired endothelial dysfunction at 6 months is supported by the lower levels of cGMP, nitrite and nitrate in the placebo treated patients, supporting the concept of impaired vascular health and endothelial dysfunction in these patients. Higher levels of MCP-1 have also been shown to predict adverse events and reflect elevated levels of inflammation (Martinovic et al., 2005), which were again seen in the placebo group at 6 months, as were higher levels of NT-proBNP. NT-proBNP, a circulating hormone released in response to increased cardiac wall stress, has been shown to be a strong predictor of cardiovascular events and congestive heart failure but has also been associated with endothelial dysfunction in patients with coronary artery disease (Huang et al., 2006). A study by Huang and colleagues has shown that endothelial vasodilator dysfunction in patients with suspected coronary artery disease is associated with elevated hs-CRP, MCP-1, and NT-pro-BNP compared to patients with a normal chronotropic response to exercise and no endothelial dysfunction. Therefore this combination of factors shown at 6 months in the nitrite group; reduced platelet reactivity, lower levels of NT-pro-BNP, hs-CRP and MCP-1 levels coupled with higher levels of nitrite/nitrate and cGMP are likely to reflect reduced inflammation, improved endothelial function and vascular health in these patients which is reflected by the reduced rate of major adverse cardiac events seen in the cohort at this time-point.

#### **6.4 Does prolonged nitrite treatment alter endothelial, or vascular smooth muscle cell growth under normoxia, hypoxia and I/R injury**

The 3<sup>rd</sup> main aim of this thesis was addressed in chapter 5 where it was demonstrated that sodium nitrite enhances EC proliferation, viability (HCAECs and HUVECS) and the wound healing of ECs (HCAECs) in hypoxic conditions at physiological concentrations. In comparison, similar physiological concentrations of nitrite resulted in a concentration-dependent inhibition of SMC (HmCASMCM) proliferation under the same hypoxic conditions with no adverse effect on cell viability. Moreover the studies revealed that sodium nitrite appears to be metabolized to biologically active NO by XOR to limit SMC proliferation via a p21-dependent mechanism and that both XOR/eNOS metabolise nitrite to active NO to promote endothelial cell growth/viability by inhibiting apoptosis and promoting proliferation by a p21 dependent mechanism. These effects appear to be cGMP dependent with elevation of cGMP seen in the treated cells compared to control and with the addition of sGC inhibitors both blocking the effect of nitrite on cell proliferation but also inhibiting the rise seen in cGMP.

Under hypoxic conditions there were distinct responses to nitrite by the different cell types. The distinct pattern of activity is likely due to the differing effects NO exerts on the cell types following its conversion from nitrite under the hypoxic conditions. This is supported by the elevation in cGMP seen with nitrite treatment in both cell (HCAECs and HmCASMCMs) types. Evidence exists that NO stimulates EC proliferation and the regeneration of healthy endothelium (Metaxa et al., 2008; Ziche et al., 1997; Cooke and Losordo, 2002) whilst NO conversely



limits SMC proliferation and migration via both cGMP-dependent and independent mechanisms (Zuckerbraun et al., 2010; Zuckerbraun et al., 2007). These effects were seen with nitrite treatment in the respective cell types which coupled with the elevation of cGMP would support the notion that nitrite under hypoxia was converted to NO to elicit these cGMP dependent effects.

#### **6.4.1 Nitrite inhibits apoptosis in endothelial cells**

The treatment of ECs (both HCAECs and HUVECs) was associated with decreased hypoxia-induced apoptosis with this reduction in apoptosis appearing to drive the increased EC number. As discussed in chapter 1 section 13, high levels of NO have been shown to induce apoptosis whereas lower levels of NO function as an important inhibitor of apoptosis by interfering with signal transduction pathways that control apoptotic cell death (Dimmeler and Zeiher, 1997; DeMeester et al., 1998; Tzeng et al., 1997; Rossig et al., 1999). Despite this the effect of nitrite on endothelial cell apoptosis is less well established but the data that does exist is consistent with the inhibitory effect demonstrated in our studies. Studies mimicking I/R injury in mouse lung vascular endothelial cells have demonstrated that nitrite (50 $\mu$ M) treatment significantly decreases I/R induced apoptosis (Sugimoto et al., 2012). Whilst other studies looking at the effect of UVA irradiation on rat aortic endothelial cells have demonstrated that nitrite (1-10mM) reduced UVA induced apoptosis (Suschek et al., 2003).

The down-stream targets of NO beyond cGMP that mediate these anti-apoptotic effects are still debated. For endothelial cells NO is thought to inhibit apoptosis by

targeting a number of distinct pathways. These anti-apoptotic effects include inhibition of caspases, increase of HSPs (heat shock proteins) and the anti-apoptotic B-cell lymphoma-2 protein (Bcl-2), nitration of p53 and activation of Akt/PKB pathway, which induces cytoprotective gene expression through NF- $\kappa$ B activation (for review see (Leon et al., 2008)). Nitrite specifically has been shown to protect endothelial barrier function against hypoxic challenge by regulating caspase-3 activity (Lai et al., 2011). Studies in zebrafish and murine ECs showed that nitrite promotes S-nitrosation and consequent inactivation of caspase-3, leading to a sustained barrier function through better maintenance of cadherin junctions (Lai et al., 2011). Whether caspase 3 was likewise affected in this study is uncertain and warrants investigating.

In addition to the decrease in apoptosis of ECs following nitrite treatment in hypoxia there was also a decrease in expression of the cell cycle regulator p21. This is consistent with previous studies that have also demonstrated that p21 levels are down-regulated with increased cell proliferation in nitrite treated neutrophilic cell line HL-60. In these cells it was shown that a similar dose of nitrite (3 $\mu$ M) administered for 24 hours resulted in a significant augmentation of the S phase of the cell cycle, an effect associated with reduction in p21 levels (Kumar et al., 2011). These effects were also shown to be NO mediated with effects blocked by NO scavengers. The results of chapter 5 are therefore consistent with the literature and do suggest that the enhanced EC proliferation seen with nitrite treatment in hypoxic conditions appears to be due to both reduced levels of apoptosis and enhancement of cellular proliferation through inhibition of p21 expression.

#### **6.4.2 Nitrite inhibits VSMC growth under hypoxic conditions**

In chapter 5, it was demonstrated that nitrite inhibited VSMC growth under hypoxic conditions. This is consistent with other studies that have shown an inhibitory effect of NO on VSMC growth. Early studies with NO donors demonstrated that NO donor agents inhibit the proliferation of isolated rat and rabbit VSMCs in tissue culture (Kariya et al., 1989; Garg and Hassid, 1989; Assender et al., 1992; Mooradian et al., 1995). Despite these documented inhibitory effects of NO on VSMC proliferation, not all studies have found an inhibitory effect of nitrite on VSMC growth. A study by Vavra and colleagues demonstrated that neither nitrite nor nitrate inhibited rat aortic VSMC proliferation in vitro (Vavra et al., 2011). The reason for this lack of effect may relate to the fact that activity was assessed under normoxic conditions and perhaps some hypoxia is required to enhance nitrite reduction.

The mechanism underlying the nitrite-associated reduction in proliferation in VSMC is different to those by which nitrite promoted EC growth. Whilst it was demonstrated that nitrite promoted EC proliferation during hypoxia by reducing cell apoptosis, an opposing increase in apoptosis did not appear to be the case in VSMC. In the VSMCs despite slightly increased numbers of apoptotic cells associated with nitrite treatment these levels were not significantly elevated. Apoptosis of VSMCs has an important role in the prevention of neointimal hyperplasia with studies showing that NO induces apoptosis. Pollman and colleagues (Pollman et al., 1996) showed that the administration of NO donors S-nitroso-N-acetyl penicillamine or sodium nitroprusside to cultured rabbit VSMC

caused apoptosis in a concentration-dependent fashion as measured by PI staining. Also in 1996, Nishio and colleagues (Nisoli et al., 2005) exposed rabbit VSMC to S-nitroso-N-acetyl penicillamine and showed a concentration-dependent increase in apoptosis by NO as measured by terminal deoxynucleotide transferase-mediated dUTP biotin nick end-labeling (TUNEL). The data shown in chapter 5 is different with no increase in apoptosis seen, which may relate to differences in coronary artery VSMC compared to aortic VSMC or reflect that low levels of apoptosis were seen in each of the VSMC experiments/treatments and therefore a greater number of experiments may be required to see a difference. It therefore appears that the inhibition of VSMC proliferation was due to a different mechanism.

#### **6.4.3 Inhibition of VSMC proliferation is dependent on p21 expression**

We demonstrated that nitrite induced inhibition of VSMC proliferation was both mediated by cGMP and dependent on increased expression of the cyclin-dependent kinase inhibitor p21. This is consistent with previous studies that demonstrate a central role for p21<sup>Waf1/Cip1</sup> protein in NO-mediated inhibition of smooth muscle cell proliferation (Ishida et al., 1997; Kibbe et al., 2000; Tanner et al., 2000; Bauer et al., 2001). Recently data has emerged that the effects of nitrite on smooth muscle cell proliferation are also NO mediated, and induce p21 expression. Studies in rat pulmonary artery smooth muscle cells (PASMC) demonstrated that sodium nitrite inhibited hypoxia-dependent PASMC proliferation in vitro, an effect associated with an increase in p21<sup>Waf1/Cip1</sup> protein levels. Additionally, nitrite failed to inhibit proliferation in PASMCs that were

deficient in p21<sup>Waf1/Cip1</sup> (Zuckerbraun et al., 2010). Further studies in rat thoracic SMCs also showed that sodium nitrite was metabolized to NO and limits VSMC proliferation via a p21<sup>Waf1/Cip1</sup> dependent-mechanism (Alef et al., 2011).

Both of these studies also provided direct evidence that XOR is critical for the reduction of nitrite to NO. Zuckerbraun and colleagues demonstrated that inhibition of XOR by allopurinol in PASMC proliferation studies diminished the antiproliferative effects of nitrite by 70%. Similarly, in their in vivo model of pulmonary arterial hypertension, supplementation with tungsten, which inhibits XOR activity by replacing molybdenum in the active site, reversed the protective effects of nebulized nitrite on the development of pulmonary arterial hypertension indicating a critical role for XOR. In similar experiments Alef and colleagues demonstrated that the use of either allopurinol or tungsten reversed the anti-proliferative effects of nitrite in rat models of Intimal hyperplasia (IH). Both of these studies demonstrate similar inhibitory effects on nitrite reduction with XOR inhibitors (70%) which are identical to the levels seen in our studies which together strongly suggest that in VSMCs and specifically intimal hyperplasia (where high levels of XOR have been demonstrated) XOR is the crucial nitrite reductase.

#### **6.4.4 Different nitrite reductases appear important in different cell types**

The effect of nitrite on HmCASMcs is likely to be due to its conversion to NO by XOR as discussed above (with no eNOS present on HmCASMcs) with existing data suggesting the importance of XOR from other studies. However as discussed in

chapter 1, the metabolism of nitrite to NO can occur by several mechanisms, including acidic disproportionation along oxygen and pH gradients, as well as by enzymatic reduction by other proteins in addition to XOR including eNOS deoxyhemoglobin and deoxymyoglobin (Cosby K, 2003; Shiva et al., 2007a; Huang et al., 2005; Lundberg JO and Weitzberg E, 2008). In our experiments the concentration-dependent increase in cell proliferation and viability caused by nitrite administration in both HUVEC and HCAECs was blocked by either L-NMMA or allopurinol implying roles for both XOR and eNOS in the conversion of nitrite to NO. The loss of efficacy was similar with both L-NMMA and allopurinol intimating that both eNOS and XOR are central nitrite reductases in the hypoxic environment and perhaps work in a synergistic manner with near complete reversal of nitrite effect with either inhibitor. This is not the first time that it has been shown that both eNOS and XOR could be important nitrite reductases, recent studies looking at models of pulmonary hypertension have demonstrated similar outcomes (Baliga et al., 2012)

#### **6.4.5 Nitrite had no effect on cell proliferation or viability in normoxia**

We showed that under normoxic conditions nitrite (0.1-10 $\mu$ M) did not affect endothelial or smooth muscle cell proliferation or viability. Nitrite is accepted as an endocrine reservoir of NO that can be reduced to bioavailable NO as oxygen concentrations and pH decrease over a physiological gradient (Lundberg JO and Weitzberg E, 2008) Previous studies have shown that it takes high concentrations of nitrite (>1-200 $\mu$ M) to have physiological effects under normoxic conditions (Rassaf et al., 2002; Lauer et al., 2001). It is under conditions of low pH and

oxygen tensions that nitrite at physiological levels (low as 100nM) exerts biological effects following its conversion to bioactive NO (Modin et al., 2001), with the first set of studies from Zweier's laboratory observing a 100-fold increase in the generation and accumulation of NO from nitrite under the acidic and highly reduced conditions of the ischaemic myocardium (Zweier et al., 1995). Virtually all studies to date showing authentic NO generation from nitrite are conducted under anaerobic conditions. However there is evidence that nitrite exerts changes in vascular tone under physiological conditions (Cosby et al 2003; Dejam et al 2007; Maher et al 2008) It is possible that in terms of vasodilation the downstream signaling pathways are more sensitive to the relatively small levels of conversion of nitrite under such conditions. The lack of any effect of nitrite on cell viability and proliferation in the present study may simply be a reflection of the differences in sensitivity of the pathways involved in the distinct effects of nitrite on cell growth/proliferation and viability compared to vasodilation.

#### **6.4.6 NO, Nitrite, intimal hyperplasia and restenosis in man**

Whether the effects of nitrite on cell growth might be of use following I/R injury or stent implantation in humans is unknown. The proliferation of VSMC is accepted as a common event in the pathophysiology of intimal hyperplasia and restenosis (Ross, 1993; Vogt et al., 2008). Delivery of NO in the form of L-arginine, pharmacological NO donors, inhaled NO, or overexpression of NOS proteins have been shown in pre-clinical models to inhibit proliferation of VSMCs and reduce the injury responses within the blood vessel wall (Napoli et al., 2001; Kibbe et al., 2000; Napoli et al., 2013). Consistent with these observations, VSMCs

transfected with eNOS showed inhibition of cell proliferation and of key cell cycle regulatory molecules (Sharma et al., 1999). Unfortunately, administration of systemic NO has not consistently demonstrated inhibition of neointimal hyperplasia in human studies. The Angioplasic Coronaire Corvasal Diltiazem (ACCORD) study showed that patients undergoing angioplasty who received NO from intravenous linsidomine while an inpatient, followed by oral molsidomine as an outpatient for a total of 6 months, had an improvement in angiographic results, with a 10% reduction in luminal diameter (Lablanche et al., 1997). However, in a separate study in patients receiving high-dose oral molsidomine for 6 months after coronary angioplasty; no effect on angiographic restenosis rate was found (Wohrle et al., 2003). In addition to the conflicting results in human clinical trials, NO administered systemically can have the undesirable effects of vasodilation, hypotension, headaches, and tolerance, ultimately limiting its clinical application. Therefore, alternative sources of NO are desirable and the delivery of NO in the form of a stable reservoir such as nitrite or nitrate is an attractive solution to this problem. It has been demonstrated that low dose sodium nitrite given prior to balloon injury in vivo prevents vascular intimal hyperplasia in both rat and mouse injury models (Alef et al., 2011). Moreover, it has been shown that nitrite delivered after the establishment of intimal hyperplasia could reverse the pathological process (Alef et al., 2011). In addition to these effects on cellular proliferation recent evidence also suggests that indices of restenosis (incl. late loss and target vessel revascularisation) can be further reduced by additional pharmacotherapy that is anti-inflammatory and anti-platelet in activity (Lee et al., 2008). Data in this thesis suggests that nitrite displays all these properties with convincing in-vitro evidence of anti-platelet and



anti-inflammatory effects displayed in chapter 4 and important anti-proliferative effects on VSMCs and pro-angiogenic effects on endothelial cells shown in chapter 5, in all an ideal combination to prevent restenosis and a strong rationale to translate this therapy into man. Thus evidence of the efficacy of sustained systemic nitrite levels post primary PCI would be of value and therefore a trial designed to test this would be important. Longer follow-up of the NITRITE-AMI patients is ongoing with both 2 and 3-year MACE rates being collected with this data available in the future.

## **6.5 Limitations**

NITRITE-AMI was powered based on the enrolment of all-comers to prevent any treatment delay and to test the therapy in as broad a group as possible. Therefore the assessment of infarct size by TIMI flow subgroups was not pre-defined. However despite this sufficient numbers of patients with TIMI flow  $\leq 1$  were available to conduct powered statistical analyses. For the secondary CMR endpoints, the study was powered for single statistical comparisons, we did not conduct multiple testing or correct for multiple comparisons.

Infarct size is an intermediate outcome measure that is commonly used to assess cardioprotective strategies in STEMI patients. However, as an intermediate outcome measure this does not provide clear understanding on hard outcomes such as MACE. The study was not powered to detect changes in MACE and although evidence of benefit was seen the low number of events prevent drawing of any reliable conclusion. This data suggests strong support for conducting a

phase 3 study in patients with TIMI $\leq$ 1 flow at point of revascularisation assessing the therapeutic potential of intra-coronary nitrite administration with MACE as the primary outcome measure.

The results regarding the potential mechanisms underlying the effect of nitrite on infarct size are interesting and raise important questions regarding these effects. However these studies were not powered to detect statistical significance and should be seen as hypothesis generating.

## **6.6 Future work**

The data in the thesis suggests that intracoronary nitrite may have a therapeutic role in the treatment of AMI that warrants further investigation in larger phase III studies, which are powered by major clinical endpoints rather than surrogate endpoints (e.g. infarct size). There are important considerations that need to be addressed in the design of this study. The first of which is to determine the primary endpoint. In terms of designing larger clinical outcome studies, it is crucial to choose clinical events e.g. major adverse cardiac events (MACEs), which are relevant to the infarct size limiting effects of the study intervention. In this regard, the combined rates of cardiac death and hospitalization for heart failure are most relevant to infarct size limitation in STEMI patients as a combined primary study endpoint, and would be the ideal outcome measure in the majority of cardioprotection trials. However as this thesis has demonstrated intracoronary nitrite has beneficial effects on the rates of coronary revascularization and non-fatal MI and therefore these should also be included in the MACE endpoint of the

phase III study. This endpoint would therefore include cardiovascular death, hospitalisation for heart failure, target vessel revascularization and recurrent MI.

As demonstrated in this thesis, for nitrite to be most effective against myocardial I/R injury administration should occur while the culprit artery is occluded and prior to myocardial reperfusion, as in the TIMI flow  $\leq 1$ . This is similar to other cardioprotective agents such as remote ischaemic conditioning where patients presenting with an occluded culprit artery accrued the most benefit in terms of infarct size reduction (Botker et al., 2010), although occasional studies have shown an overall benefit in all comers irrespective of TIMI flow (Botker et al 2010). On this basis, for clinical cardioprotection studies, it is advisable to only include those STEMI patients with an occluded culprit artery and therefore for the phase III trial, the inclusion criteria should specify that only patients with occluded vessels at the time of angiography (TIMI $\leq 1$ ) should be included.

Despite the primary endpoint being MACE, it would be important to assess cardiac function, myocardial area at risk and markers of both infarct size and reperfusion injury. Therefore patients should undergo CMR scanning in addition to the assessment of the clinical endpoints. The timing of this scan or scans is debated. For NITRITE-AMI delayed enhancement infarct size was assessed at both 2 days and 6 months after infarction. There are advantages and disadvantages of both these timepoints, with both shown to be associated with histopathological assessed infarct size and both associated with prognosis (Kim et al, 2009). However the early scan may overestimate infarct size due to the presence of odema whereas a late scan has been shown to potentially

underestimate infarct size due to shrinkage (Kim et al 2009). The other advantage of performing an early (2 day) scan is that it provides an assessment of the area at risk for calculation of myocardial salvage. For the phase III study we would therefore perform an early scan at 2-3 days to enable an assessment of myocardial salvage and infarct size which although may overestimate infarct size it would do so for both treatment groups and provides comparison to other studies (Piot et al 2008).

Another major confounding factor for cardioprotective strategies is concomitant medication, which patients are on for their cardiovascular risk factor, co-morbid condition, or as part of the treatment of the ongoing acute MI. These pharmaceutical agents have been shown in pre-clinical and clinical studies to either block the cardioprotective effect (for example, certain oral anti-diabetic sulphonylureas, nitrates when nitrate tolerance develops, certain statins) or induce cardioprotection themselves. Specifically for nitrite it has been suggested that the prior use of organic nitrates (e.g. GTN) may induce a degree of cardioprotection limiting the potential for additional cardioprotection by nitrite. This fact has been raised by 2 recent clinical studies, the first of which being the NIAMI study where 90% of patients had received organic nitrates acutely prior to reperfusion and it was suggested the lack of benefit of intravenous nitrite may be due to this prior cardioprotection (Siddiqi et al 2014). The 2<sup>nd</sup> recently presented study, NOMI (Nitric Oxide for inhalation to reduce reperfusion injury in acute ST-elevation Myocardial Infarction) where although no difference in infarct size was seen between patients who received inhaled nitric oxide and those who did not (18% vs 19.4%,  $P=0.44$ ) they found in a pre-specified sub-group analysis of

patients who had received intracoronary or intravenous nitroglycerin (IC/IV NTG) a significant interaction ( $P=0.014$ ) with the use of inhaled nitric oxide. Among NTG-naïve patients ( $n=132$ ), nitric oxide inhalation was associated with significantly smaller infarcts compared to patients who had previously received NTG ( $n=93$ ) (Janssens 2014). Although it has been suggested that the use of organic nitrates could be an important reason for the discrepancy seen between the preclinical data and the clinical data in these 2 studies, new therapies need to be effective when given to patients receiving standard therapy and therefore patients should not be excluded from the study based on acute organic nitrate use. However pre-specified sub-group analysis of patients who had received intracoronary or intravenous nitroglycerin will be included.

## **6.7 Conclusions**

In summary the infusion of intra-coronary nitrite appears safe when delivered during primary PCI for AMI with no documented adverse effects associated with the drug. However when delivered in all-comers, it had no significant effect on infarct size but did result in reduced MACE at 1 year. In patients with TIMI flow  $\leq 1$  there was a significant reduction in cardiac enzyme release and infarct size, with reduced microvascular obstruction and an increased myocardial salvage index demonstrated on CMR, suggesting a potential beneficial effect when delivered in this group of patients.

Mechanistically, levels of platelet reactivity were significantly reduced after primary PCI in the nitrite compared to the placebo group, as were levels of the

inflammatory mediators hs-CRP and MCP-1. These markers were associated with infarct size and therefore are likely to reflect reduced levels of systemic inflammation associated with this reduction in infarct size. Importantly these decreases in the nitrite group were maintained out to 6 months after reperfusion suggesting improved outcomes irrespective of aetiology. In nitrite treated patients the elevation of neutrophils associated with AMI was suppressed compared to placebo and this was specifically associated with a decrease in the level of activation markers involved in adhesion, CD11b and the neutrophil chemokines (CXCL-1 and CXCL-5).

Nitrite appears to protect ECs from the damaging effects of ischaemia or I/R injury but exerts an opposing repressive influence over SMC growth, suggesting that in an environment where improved EC growth but repressed SMC growth is desired, such as in restenosis following stent implantation, raising circulating nitrite levels may be of some therapeutic utility.

Together this data suggests that intracoronary nitrite may have a therapeutic role in AMI that warrants further investigation in larger phase III studies with separate phase II studies addressing the use of nitrite to prevent restenosis following PCI also justified.

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## 8.0 Appendix

### 8.1 Summary Patinet information sheet (PIS)

#### **NITRITE- AMI Trial**

**A randomised, double-blind, placebo-controlled trial assessing the safety and efficacy of intracoronary nitrite infusion during acute myocardial Infarction**

**Protocol number 2.1**

#### **Summary Information Sheet**

**This is a summary of the research study. Please read the attached information which explains the study in more detail before making the decision whether or not to take part.**

- You are invited to join a research study, recruiting patients having a heart attack and being admitted via the Heart Attack Centre. This study involves a new treatment, which we hope will limit damage to the heart muscle caused by a heart attack.
- If you agree to take part in the study, the angioplasty procedure to treat your heart attack, will go ahead as usual however we will also inject a drug down the affected artery at the time of the procedure.
- We will inject either a drug called sodium nitrite or saline (placebo) down the affected artery and into the damaged tissue. The drug is designed to prevent some of the damage that will continue to occur despite the artery being opened. This may or may not be effective
- Sodium nitrite is a source of the chemical nitrite, which is found occurring naturally in your blood and tissues. During a heart attack it acts as a source of nitric oxide, which is essential for blood vessel function.
- The trial is randomised, so you will be randomly allocated to receive either the sodium nitrite or placebo. There is a 50/50 chance of you being allocated to either group.
- Following the treatment, you will return to the ward and receive standard care for all patients having had treatment for a heart attack, aiming for discharge as usual between 2 - 5 days.
- There are no known major adverse effects from sodium nitrite being given in this situation however as this is a first of its kind study there may be potential risks that are unknown.
- The study will involve:-
  - Extra blood tests and ECGs (electrical heart tracings) during your hospital stay
  - a clinic visit at 6 months
  - yearly telephone calls for the next 3 years.

**Thank you for taking the time to read this. Please ask if there are any questions**

## 8.2 Detailed Patient information sheet (PIS)



**The London Chest Hospital,**  
Barts Health NHS Trust,  
Bonner Road,  
London,  
E2 9JX

### Patient Information Sheet

#### **NITRITE-AMI:- A randomised, double-blind, placebo-controlled trial assessing the safety and efficacy of intracoronary nitrite infusion during acute myocardial infarction**

When you arrived at the London Chest Hospital having your heart attack you kindly agreed to participate in a research study. This research study consists of 2 parts; the 1<sup>st</sup> of which you have already agreed too and has started. The purpose of this patient information sheet is to provide more information about this 1<sup>st</sup> part of the study that you have already agreed to participate in and also to explain details about a 2<sup>nd</sup> optional part of the study.

One of our research team will go through the information with you to make sure that you understand. Ask us if there is anything that is unclear or if you would like more information or time.

Please take time to read the following information carefully. Talk to others about the study if you wish.

- **Part 1 tells you about the 1<sup>st</sup> part of the study, explaining what has happened already and what will happen in the future**
- **Part 2 explains about the 2<sup>nd</sup> part of the study and what will happen to you if you agree to take part**

#### **Why are we doing this research?**

Despite advances in the treatment of heart attacks such as balloon treatment to the blocked artery (primary angioplasty), the complications and death rates from failure of the heart to pump adequately and from heart rhythm problems remain high. The size of

the heart attack is the major reason for these adverse outcomes. Whilst re-opening the artery allows the blood to flow to the area of the heart starved of blood and oxygen, this process also causes considerable damage itself (called “reperfusion injury”) and increases the size of the heart attack. The aim of this study is to decrease the amount of this reperfusion injury and subsequently reduce the size of the heart attack. We aim to do this by giving a substance called sodium nitrite down the damaged artery and into the tissue. This has been shown in models of a heart attack to decrease reperfusion injury and the size of heart attacks.

### **Why was I chosen?**

You were chosen because you were admitted as an emergency with a heart attack (‘myocardial infarction’), and underwent an **emergency** coronary balloon angioplasty and stent insertion

This study aims to recruit 80 similar people who are having emergency coronary balloon angioplasty and coronary stents insertion within Barts and the London NHS trust within the next 2 years.

## **Part 1**

### **What has happened to me so far?**

This research study involves the use of sodium nitrite to try and limit the damage to the heart muscle caused by a heart attack. To test whether this works this study is ‘randomised’. This means that half of the patients will receive nitrite and the other half will receive a ‘placebo’ or ‘dummy’. The placebo used for this study will be saline (water). The decision is made in a way similar to ‘flipping a coin’ and neither you nor anyone involved in your care will know which treatment you have received. This enables us to compare both groups to assess the effectiveness of this new treatment. The trial process will be exactly the same for all patients enrolled in the study.

Therefore during the PCI procedure either the sodium nitrite or placebo (saline) was injected down your damaged artery over a period of 30 seconds as the artery was being opened.

Following this procedure we have so far taken regular blood tests, which will help monitor the condition of your heart (a total of 6 extra teaspoons of blood will be taken in addition to the routine blood samples taken before your treatment (already taken) and at discharge).

### **What is Sodium Nitrite?**

Sodium nitrite is a source of nitrite, a substance that is found naturally in your blood and tissues. It is an important store of nitric oxide (NO), which is essential for maintaining the blood vessel in a normal healthy state. It maintains the blood vessel diameter (by relaxing the muscle in the vessel) and prevents blockages by stopping white blood cells from crossing the endothelial wall and by stopping platelets from sticking to the blood vessel wall. During a heart attack, we know that levels of nitric oxide are reduced which removes these helpful effects. In situations like this, sodium nitrite can be used to replace these low levels of nitric oxide. It has been shown in



animals to help decrease the damage caused by a heart attack but up until now no study looking at whether it works in humans has been performed, that is the purpose of this study.

### **What happens to me during the rest of the study?**

During the rest of your hospital stay and throughout the study, you will be followed up closely. This will include electrical tracings of your hearts activity – Electrocardiogram (ECG) and blood tests, which will help to assess the health of your heart and regular assessments by your doctor. The ECG will involve small metal electrodes stuck onto your arms, legs and chest. Wires from the electrodes are connected to the ECG machine. The machine detects and amplifies the electrical impulses that occur at each heartbeat and records them on to a paper or computer. A few heartbeats are recorded from different sets of electrodes. The test takes about five minutes to do.

At 6 months you will need to attend the hospital to have a physical examination, a blood test (4 teaspoons of blood), and a further heart tracing (ECG)

Following this, you will be contacted by telephone at 12 months and then at 2 years to ask about your health.

### **Are there any long-term risks to my health of having the treatment?**

The sodium nitrite treatment itself is not associated with any major side effects or risks, however as this the first of this kind there may be potential risks that are unknown. The delivery of this drug during the procedure did not lead to any significant changes in the standard therapy being administered.

### **What are the benefits from taking part?**

You have already kindly agreed to participate in the 1<sup>st</sup> part of the research study where the treatment has been given. Studies performed in animals have shown that this treatment may lead to a decrease in the size of the heart attack that you suffered, however this is the first study to look at its use in people like yourself so we don't know if this effect will occur. There is also a 50/50 chance that you received placebo/saline and therefore there may or may not be any benefit to taking part in this study. You will however have received the standard optimal treatment for your condition.

### **What will happen if I don't want to carry on in the study?**

You are under no obligation to take part in this study and are free to withdraw at anytime with no reason, this will not affect the standard of care you receive. If you do decide to withdraw from the study we will still need to use the data collected up to your withdrawal

### **Will my taking part in this study be kept confidential?**

Data will be collected about your medical history, investigations, treatments and any complications. All information collected during the research will be kept strictly confidential.

Data will be stored in a locked room on a secure computer database. Identifiers such as name, address, hospital number will not be used.

Your records may be reviewed by staff from Barts and the London NHS Trust to ensure that the data collected is accurate for the purposes of analysing the results. Your records may be reviewed by people from the regulatory authorities to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

### **What if something goes wrong?**

Barts and the London NHS Trust has agreed that if you are harmed as a result of your participation in the study, you will be compensated, provided that on balance of probabilities, an injury was caused as a direct result of the procedures you received during the course of the study. These special compensation arrangements apply where an injury is caused that would not have occurred if you were not in the trial. If you are harmed due to someone's negligence, then you may have grounds for a legal action as with all NHS care.

### **What happens to the results of the research study?**

When the research is completed and the results analysed, our findings will be presented at scientific conferences and published in scientific journals. Your identity will not be divulged in any publication, presentation or report. We are happy to provide you with a copy of any publication of the findings of the study at your request.

### **Who is organising and funding the research?**

Barts and the London NHS Research and Development Department is the sponsor of this study and is responsible for ensuring all aspects of the study are carried out to the highest standard. The research is funded by a grant received from Barts and the London Charities.

### **What if new information becomes available?**

Sometimes new information becomes available during the course of a research project that is directly relevant to participants within the research. If significant new information came to light that had a bearing on the study and your participation, then this information would be considered by a committee who oversee the conduct of the trial and you would be informed.

### **What happens when the research study stops?**

When the study is completed you will not be required to continue with any of the study procedures and your care will continue as usual.

This completes Part 1 of the information sheet. Please continue to read the additional information in Part 2 regarding the 2<sup>nd</sup> part of the study to decide if you wish to also undertake it.

## **Part 2**

### **2<sup>nd</sup> part of study**

Before deciding whether to also take part in the 2<sup>nd</sup> part it is important for you to understand what it will involve. Please take time to read the following information carefully. Talk to others about the study if you wish. This will have no bearing on the 1<sup>st</sup> part of the study, this will proceed as discussed.

#### **Do I have to take part?**

NO. It is up to you to decide whether or not to take part. The purpose of this information sheet is to help you make a decision, a member of the research team will go through any details you wish explaining. If you decide to take part you will be asked to sign a second consent form. You are free to withdraw from this part or the 1<sup>st</sup> part of the study at any time without giving a reason. A decision to participate, not participate or withdraw from the study will not affect your future medical treatment.

#### **What will happen to me if I take part?**

Before discharge from hospital (at least 2 days following your procedure), you will undergo a cardiac magnetic resonance scan (CMR) scan. This will further assess the pumping ability of your heart. At 6 months when you come back to the hospital for the clinic visit for part 1, you will have another CMR scan. This will be identical to the 1<sup>st</sup> scan.

#### **What does the CMR scan involve?**

Cardiac magnetic resonance scans (CMR) use a magnet, radio-waves and a computer to take detailed pictures of the inside of your body. It does not use x-rays, is painless and safe. It involves lying down on a bed and being taken into a scanner shaped like a tunnel. Inside the scanner you will be asked to lie still in order to get clear pictures. You will not feel anything. During the scan you hear a rhythmic banging noise. This is quite normal. The radiographer will be in contact with you throughout the examination, which typically takes 30-60 minutes and will require for you to have an injection of contrast through a cannula in your arm.

#### **What are the possible risks and side effects of any treatment received when taking part?**

As always, your health is our priority. If at any time you experience a recurrence of symptoms, or your condition requires a different treatment strategy, you will receive appropriate treatment regardless of the study. This research is at the forefront of our understanding and technology and as such, potential risks apart from those mentioned below, are unknown. However, given our current understanding of this technology we do not foresee any major problems. Potential risks mentioned below are minimised as procedures are performed by experienced staff.

CMR scan – the CMR scan does not use radiation. There are no major known side effects or risks attached to CMR although some patients may find it a little claustrophobic. The main issues are related to possible allergy to contrast agent and the scanning process. Most CMR exams are painless, however, some patients find it

uncomfortable to remain still during the scan, whilst others may experience a sense of being closed in (claustrophobia). Therefore, sedation can be arranged for those patients who anticipate anxiety, but fewer than one in 20 require it. You will be alone in the exam room during the procedure, however, the technologist will be able to see, hear and speak with you at all times using a two-way intercom. If you wish the scan to be stopped at any point, it will be. There is a very slight risk of an allergic reaction if contrast material is injected. Such reactions usually are mild and easily controlled by medication. If you experience allergic symptoms, a radiologist or other physician will be available for immediate assistance. You will be assigned a study nurse or doctor whom you can contact in case you have any concerns or questions about the study.

### **What are the benefits of taking part?**

You have already agreed to participate in the 1<sup>st</sup> part of this research study where the treatment or placebo has been given. There would be no symptomatic or health benefit from taking part in this 2<sup>nd</sup> part. The only benefit is that we would be able to get a more detailed look at your heart and how it pumps which we would otherwise not perform.

### **Expenses and Payments**

The costs of your transport to hospital for the 6 month clinic follow-up are already covered,

There will be no additional payment in exchange for your participation in the 2<sup>nd</sup> part of the study.

### **What will happen if I don't want to carry on in the study?**

The 2<sup>nd</sup> part of this study primarily involves two CMR scans, if you dislike the 1<sup>st</sup> scan you can withdraw prior to the 2<sup>nd</sup>, this will not affect your participation in the 1<sup>st</sup> part of the study.

### **What if there is a problem?**

If you have concerns about any aspects of this study, you should ask to speak with the research team who will do their best to answer your questions (Dr Dan Jones or Prof Anthony Mathur)

If you remain unhappy and wish to complain formally you can do this by contacting: The Patient Advisory Liaison Service (PALS) if you have any concerns regarding the care you have received, or as an initial point of contact if you have a complaint. Please telephone 0207 377 6335 or email [pals@bartshealth.nhs.uk](mailto:pals@bartshealth.nhs.uk). You can also contact PALS by asking at the hospital reception.

**Contact Details: If you require any further information please contact**

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For Information out of hours call switchboard (020 7377 7000) and ask for the on call cardiology registrar at the London Chest Hospital.

Thank you for taking the time to read this patient information sheet. If you decide to take part we will ask you to sign a consent form, of which you will be given a signed copy.

**Thank you for taking the time to read this. Please ask if there are any questions.**